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# Steric effects in vitamin B12 complexes

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# STERIC EFFECTS IN VITAMIN $B_{12}$ COMPLEXES

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A Thesis submitted to the Faculty of Science,
University of the Witwatersrand, Johannesburg
for the Degree of Doctor of Philosophy

March 1980

Declaration

I hereby declare that the work reported in this thesis was carried out exclusively by myself and that this thesis has not been submitted for a degree at any other university.

S. M. Chemaly.

Susan Mary Chemaly

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To my father and mother

ABSTRACT

The increase in steric compression in organocobalamins in the series methyl < ethyl < isopropyl; ethyl ~ n-propyl < isobutyl < neopentyl; cyclopropyl < cyclobutyl < cyclopentyl < cyclohexyl gives rise to parallel changes in the spectra, equilibrium constants and lability of the Co-C bond in organocobalamins. The spectra of the five-coordinate, protonated, 'base-off' forms show parallel changes in the 440-460nm region, in the region around 375nm and in the band at 303nm with increasing steric compression and the spectra of the six-coordinate, 'base-off' forms show a gradual shift of the αβ band from 522nm to 510nm with increasing steric compression. The pKa values and the ratio of five-coordinate, unprotonated, 'base-off' forms to six-coordinate 'base-on' forms increase with increasing steric compression. Organocobalamins show increasing ease of decomposition by homolytic fission and/or β-elimination with increasing steric compression.

It is proposed that steric compression in the organocobalamins is partially relieved by lengthening of the Co-C bond and by conversion of the six-coordinate 'base-on' complex to a five-coordinate 'base-off' complex with concomitant movement of the cobalt atom out of the plane of the corrin ring. It is shown that there are two different forms of 'base-off' organocobalamins present in neutral solution and it is suggested that benzimidazole is not coordinated in either form but is completely free in one form and held close to the corrin ring by hydrophobic bonding in the other.

The reactions of neopentylcobalamin in the presence of oxygen, alcohols, thiols and imidazole are studied and it is proposed that neopentylcobalamin undergoes homolytic fission at room temperature due to lengthening of the Co-C bond caused by steric compression. It is suggested that neopentylcobalamin can be used as a model for the enzyme-induced homolytic fission of adenosylcobalamin.

It is shown that cyclopropylmethylcobalamin rearranges to 3-butenylcobalamin and that this reaction is accelerated by increase of temperature and by light and it is therefore proposed that the formation of the Co-C bond inhibits the otherwise facile rearrangement of cyclopropylmethyl.

The enzymatic homolysis of the Co-C bond of adenosylcobalamin is discussed in the context of steric effects in organocobalamins; an enzyme-bound, sterically-distorted form of adenosylcobalamin is proposed and it is shown that such an intermediate could explain the presence of unexpected bands in the UV-visible spectra of the steady state of the enzyme reactions.

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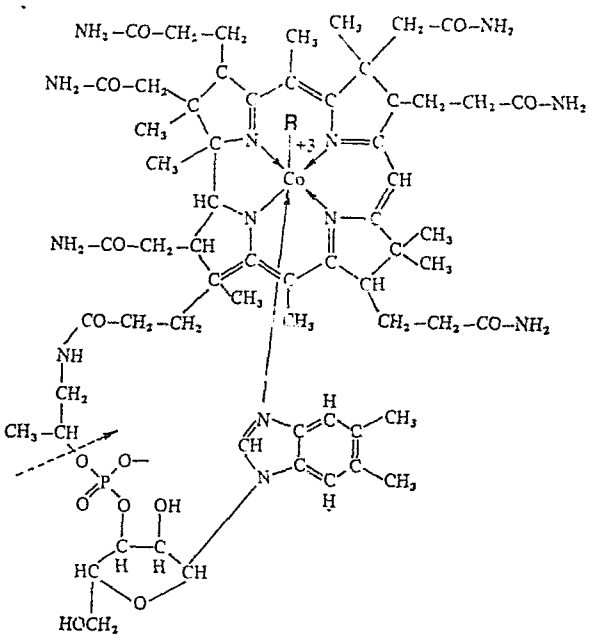

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# CHAPTER 1 - INTRODUCTION

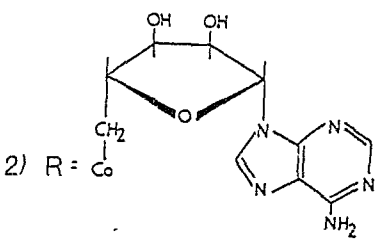
## 1.1 The history of vitamin $B_{12}$[1,2,3]

In man, deficiency or malabsorption of vitamin $B_{12}$ results in pernicious anaemia. This disease was first described early in the nineteenth century and is characterized by a decrease in the number of red blood cells and an increase in abnormalities in the red blood cells. In 1926 Minot and Murphy showed that pernicious anaemia could be alleviated by feeding patients large amounts of raw liver. This led to a search for the so-called 'anti-pernicious anaemia' factor which culminated in the isolation of crystalline vitamin $B_{12}$ in 1948 by Folkers and his co-workers and in the same year by Smith and Parker. The isolation of vitamin $B_{12}$ was made difficult by the small amounts of vitamin $B_{12}$ (<1ppm) present in liver and the lack of a suitable assay procedure (patients suffering from pernicious anaemia were in short supply). The discovery of a microbiological assay method for $B_{12}$ considerably speeded up the final stages of the isolation of vitamin $B_{12}$. Vitamin $B_{12}$ is now prepared by bacterial fermentation rather than by isolation from liver.

Synthetic and degradative methods were used to establish that vitamin $B_{12}$ contained 5,6-dimethylbenzimidazole, D-ribose and aminopropanol as well as cobalt and cyanide and suggested the presence of a macrocyclic ligand related to porphyrin. The



1) R = $CN^-$



2) R = Co

Fig.1.1 Structure of 1) vitamin $B_{12}$ (cyanocobalamin) and
2) adenosylcobalamin.
Hydrolysis at ---→ gives the corresponding cobinamides.

structure of the corrin ring was elucidated by Hodgkin, using X-ray crystallography, and the correct structure (Fig.1.1) of vitamin $B_{12}$ was proposed in 1957. In 1958 Barker discovered the coenzyme form of vitamin $B_{12}$ while studying the metabolism of glutamate in Clostridium tetanomorphum. Using X-ray crystallography it was found that the cyanide group in vitamin $B_{12}$ is replaced by an adenosyl group in the coenzyme (Fig.1.1) and that a Co-C σbond is present. Coenzyme $B_{12}$ (adenosylcobalamin) was the first naturally occurring organometallic compound to be discovered. Later methylcobalamin, which also contains a Co-C σbond, was also discovered in vivo. In 1979 the total synthesis of vitamin $B_{12}$ was reported by Woodward.[4]

## 1.2 Structure of corrinoids[5a,6,7]

Cyanocobalamin, adenosylcobalamin and methylcobalamin belong to a family of compounds known as corrinoids. Corrinoids consist of a central metal ion (usually cobalt in the +3 oxidation state), a macrocyclic corrin ring (Fig.1.2) which is coordinated via its four nitrogens to the cobalt ion, and two further ligands in the fifth and sixth coordination positions.

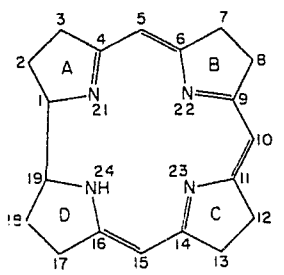



Fig.1.2 The basic corrin ligand

The corrin ligand is made up of four reduced pyrrole rings and is nearly planar. It is related in structure to the porphyrin ring but is less extensively conjugated. The numbering of the corrin ring corresponds to that of the porphyrin ring, except that the C-20 carbon atom is missing in the corrin ring. The four five-membered rings of corrin are labelled A, B, C and D. The corrin nucleus has on its periphery three acetic acid, four propionic acid, and eight methyl groups.

In cobalamins the propionic acid attached to $C_{17}$ is amidated with (R)-1-amino-2-propanol and the other carboxyl groups are converted to amide groups. All the propionamide groups point away from the plane of the ring in one direction (the 'upper' side of the corrin ring) and all the acetamide side-chains in the other direction (the 'lower' side of the corrin ring). The fifth coordination position in cobalamins is taken up by a nitrogen atom of 5,6-dimethylbenzimidazole. The other nitrogen of 5,6 dimethylbenzimidazole is bound to ribose in an α-glycosidic linkage and the ribose moeity is in turn bound, via a phosphate linkage, to the aminopropanol side chain. In cyanocobalamin the sixth coordination position is taken up by cyanide ($CN^-$). Cyanocobalamin is a six-coordinate, diamagnetic complex. The positive charges on the Co(III) ion are balanced by the negative charges on the corrin ring, the cyanide and the phosphate. Adenosylcobalamin, methylcobalamin and other organocobalamins are also diamagnetic and can be considered as complexes of Co(III) with a carbanion as sixth ligand.

Cobinamides are corrinoids in which the phosphate linkage to the ribose moiety of the benzimidazole side-chain has been hydrolysed (Fig.1.1). The coordinated benzimidazole is usually replaced by another ligand, often cyanide or water.

The numbering of the atoms of corrinoids is given in Fig. 1.3.

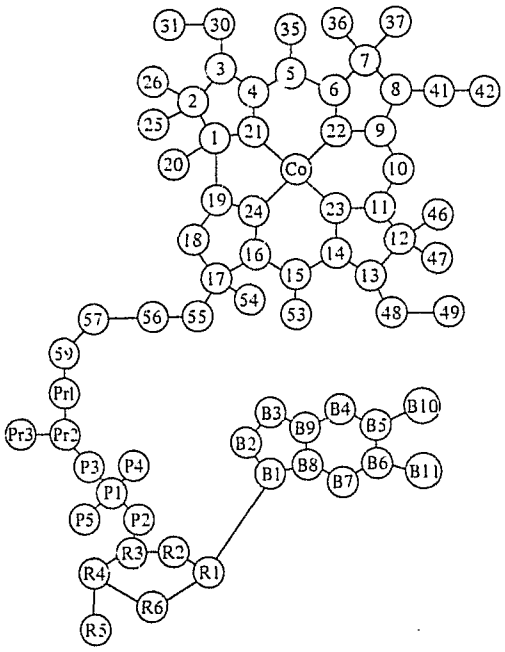



Fig.1.3 Numbering of the atoms in corrinoids

## 1.3 Nomenclature of corrinoids[8]

Cobalt corrinoids are given names based on the prefix cob (cob indicates the presence of a cobalt ion). The coordination sites on the lower (acetamide) and upper (propionamide) sides of the corrin ring are denoted by α and β. Corrinoids are usually named by writing the α-substituent first in brackets, followed by

the β-substituent and then the name of the corrinoid eg. (cyano)methylcobinamide. For cobalamins the 5,6-dimethylbenzimidazole base, whether coordinated or not, is not included in the name and cobalamin is preceded only by a term representing the β-substituent eg. cyanocobalamin. If the oxidation state of cobalt is not given the corrinoid is assumed to contain Co(III). If a corrinoid has cobalt in an oxidation state other than (III) this is specified in its name eg. cob(I) alamin, cob(II) alamin, cob(I) inamide, cob(II) inamide.

The abbreviations $B_{12}$ (cyanocobalamin), $B_{12a}$ (aquocobalamin), $B_{12r}$ (cob(II) alamin), $B_{12s}$ (cob(I) alamin), $B_{12r}$ (cobin) (cob(I) inamide) and $B_{12s}$ (cobin) (cob(I) inamide) will be used throughout this thesis.

1.4 Reduced corrinoids[5b,9]

Corrinoids containing Co(I) or Co(II) are obtained by reducing a Co(III) complex under anaerobic conditions. Examples are given, for $B_{12a}$, in the following equations:-

$$Co\text{-}OH_2 \xrightarrow[\text{alkaline solution}]{RSH} Co(II) \qquad RSH = \text{thiol}$$

$$Co\text{-}OH_2 \xrightarrow{NaBH_4} Co(I)$$

Co(I) and Co(II) corrinoids are sensitive to aerial oxidation giving Co(III) corrinoids. Co(I) corrinoids rapidly decompose in aqueous solution at low pH to give Co(II) and hydrogen, probably via an intermediate cobalt hydride. Co(II) corrinoids are low spin $d^7$ compounds with one unpaired electron. In neutral solution

$B_{12r}$ is probably 5-coordinate with the benzimidazole base coordinated to cobalt but in acidic solution the benzimidazole base is protonated and removed from coordination.[10] For $B_{12r}$ at acid pH, $H_2O$ is probably coordinated.[10] Co(I) corrinoids are spin-paired $d^8$ complexes. In $B_{12s}$ the benzimidazole base is not coordinated to cobalt.[11]

1.5 Organocorrinoids[2,5c,6]

The discovery of adenosylcobalamin and methylcobalamin acted as a stimulus for chemists to prepare these two compounds as well as other organocorrinoids. The organocobalamins and organocobinamides prepared range from those with small, simple ligands such as ethyl and vinyl to those with more complicated ligands such as 1-norbornyl.[12] All of these organocorrinoids contain a Co-C $\sigma$ bond. Sigma-bonded alkyl derivatives of metals of the first transition series are usually rather unstable and the stability of the Co-C bond occasioned some surprise when first discovered. Adenosylcobalamin and methylcobalamin are stable in the dark both in the solid state and in solution. This is the case for most organocorrinoids unless they have an organoligand which interacts sterically in an unfavourable way with the corrin ring eg. isopropylcobalamin. All organocorrinoids are unstable in the presence of light, their characteristic reaction being homolytic cleavage of the Co-C bond to give cobalt (II) and an organic free radical.

Organocobalamins and organocobinamides are generally

prepared by first reducing a cobalamin (eg. aquocobalamin, cyanocobalamin) or cobinamide (eg. cyanoaquocobinamide, diaquocobinamide) in the III oxidation state to the I oxidation state followed by reaction of the Co(I) corrinoid with compounds such as alkyl halides, epoxides, alkenes and alkynes.

Organocorrinoids undergo many types of reactions involving the cobalt coordination sphere (as distinct from the corrin ring or side-chains). The Co-C bond may be broken by homolytic fission where the intermediates are Co(II) and a free radical eg.

$$Co\text{-}CH_3 \rightleftharpoons^{light} Co(II) + R\cdot$$

by heterolytic fission eg.

$$Co\text{-}CH_2CH_2OH + H^+ \longrightarrow Co\text{-}OH_2 + CH_2 = CH_2$$

$$Co\text{-}COCH_3 + OH^- \longrightarrow Co(I) + CH_3COOH$$

or by a $\beta$-elimination reaction eg.

$$Co\text{-}CH(CH_3)_2 \rightleftharpoons Co\text{-}H + CH_3\text{-}CH = CH_2$$

The organoligand may also be reversibly or irreversibly modified without breaking the Co-C bond eg.

$$Co\text{-}C \equiv CH + Ag^+ \rightleftharpoons Co\text{-}C \equiv C\text{-}Ag + H^+$$

$$Co\text{-}C \equiv CH + H_2O \xrightarrow{H^+} Co\text{-}CO\text{-}CH_3$$

The types of reactions undergone by organocorrinoids are shown in fig.1.4.

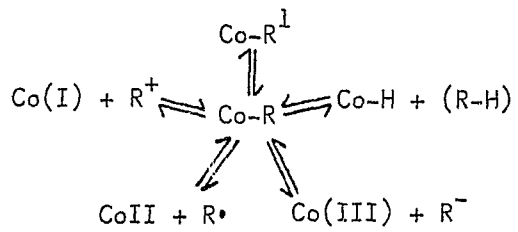



Fig.1.4 Reactions of organocorrinoids

## 1.6 Stable yellow corrinoids[5d]

Reactions of cobalamins which proceed with a change in the oxidation state of the cobalt ion often give rise to by-products which are known as stable yellow corrinoids. Generally, stable yellow corrinoids have an absorption spectrum with a band at about 460nm but, unlike $B_{12r}$ or 'base-off'organocorrinoids, they cannot be converted to typical Co(III) corrinoids by the action of light, oxygen or cyanide (separately or in combination). The formation of stable yellow corrinoids often occurs under conditions where free radicals are present. This suggests that stable yellow corrinoids are formed by free-radical attack on the corrin ring. X-ray crystallography of a stable yellow corrinoid produced by the action of ascorbic acid in oxygen on dicyanocobyrinic acid heptamethylester showed the presence of a hydroxy group at C-5 and a five membered lactor ring between C-6 and C-7.[13] It was suggested that this stable yellow corrinoid arose by attack of a hydroxyl radical on the corrin ring.[13]

## 1.7 Enzyme reactions requiring cobalt corrinoids[2,7,14 15,16]

Enzymes containing cobalt corrinoids catalyse many reactions, mainly in microorganisms. Only two enzymes occurring in higher animals have been found to require cobalt corrinoids. These are methylmalonyl-coenzyme A mutase and $N^5$-methyltetrahydrofolate: homocysteine methyltransferase. Enzyme reactions requiring cobalt corrinoids can be divided into two classes:-

1) reactions requiring adenosylcobalamin and

2) methyl transfer reactions involving methylcobalamin.

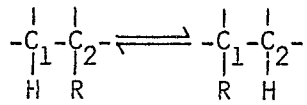
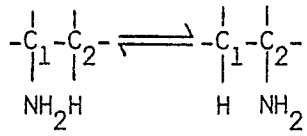
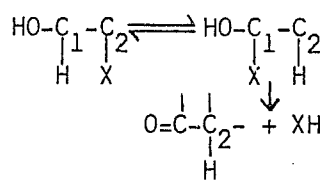
The enzymes requiring cobalt corrinoids are classified and tabulated below (Table 1.1). Only the rearrangement or isomerization reactions, (Class 1:A) in the table, will be considered in detail.

The isomerase enzyme reactions have many features in common and their reaction mechanisms appear to be very similar. For all these reactions the first step (after the binding of substrate) is the cleavage of the Co-C bond in adenosylcobalamin. The cleavage is probably homolytic and the products are $B_{12r}$ and the adenosyl free radical. The adenosyl free radical then abstracts a hydrogen atom from the substrate to give 5'-deoxyadenosine and a substrate free radical. The substrate free radical, either directly or via a carbonium ion or cobalt-substrate complex rearranges to give a product free radical which abstracts a hydrogen atom from 5'-deoxyadenosine to give the product and an adenosyl free radical. The adenosyl free radical combines w.

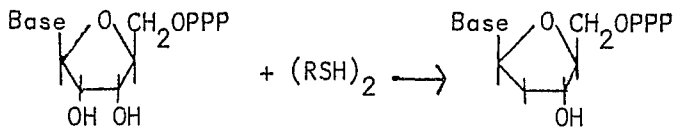
Table 1.1 - Enzyme Reactions Requiring Cobalt Corrinoids

Class 1 - Reactions requiring adenosylcobalamin

A. Rearrangement or Isomerization Reactions

| Type of reaction | | Enzyme |
|---|---|---|
| Carbon-skeleton rearrangement |  | Glutamate mutase<br>Methylmalonyl-coenzyme A mutase<br>α-Methyleneglutarate mutase |
| Migration of an amino group |  | L-β-lysine mutase<br>D-α-lysine mutase<br>D-ornithine mutase |
| Conversion of diols or amino alcohols to aldehydes |  | Dioldehydrase<br>Glycerol dehydrase<br>Ethanolamine ammonia-lyase |

B. Conversion of ribonucleotides to 2'-deoxribonucleotides

| Reaction | Enzyme |
|---|---|
|  | Ribonucleotide reductase |

$$+ (RS)_2 + H_2O$$

$(RSH)_2$ = dithiol

Class 2 - Reactions involving methylcobalamin

| Reaction | |
|---|---|
| $5\text{-}CH_3\text{-}THF + HS(CH_2)_2CH(NH_2)COOH \longrightarrow$ | $N^5$-methyltetrahydrofolate: |
| $THF + CH_3S(CH_2)_2CH(NH_2)COOH$ | homocysteine methyltransferase |
| $5\text{-}CH_3\text{-}THF + HS(CH_2)_2SO_3 \longrightarrow THF + CH_3S(CH_2)_2SO_3$ | "Methane synthetase" |
| $5\text{-}CH_3\text{-}THF + CO_2 \longrightarrow THF + CH_3COO^-$ | "Acetate synthetase" |

THF=tetrahydrofolate

$B_{12r}$ to regenerate adenosylcobalamin. Thus, the hydrogen transfer reaction is intermolecular but the transfer of the larger group is intramolecular. The mechanism of the rearrangement reactions is represented schematically below (Fig.1.5).

The experimental evidence in favour of scheme 1.5 is summarized in Table 1.2.

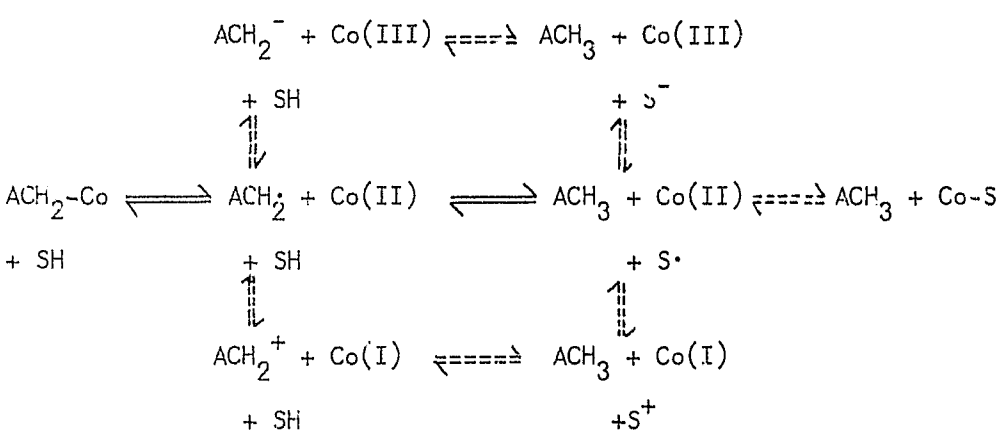



Fig.1.5 Scheme of possible reaction mechanisms for the isomerase enzymes.

We are still a long way from understanding the reaction mechanism of adenosylcobalamin-dependent rearrangement reactions and the specific role of the apoenzyme is still obscure.

## 1.8 Aims of this thesis

The work described in this thesis was undertaken with the following aims:-

1) To elucidate the role of steric factors, as opposed to electronic factors, in determining the spectra, equilibria and

reactions (specifically cleavage of the Co-C bond) of alkylcobalamins. In order to do this three series of alkylcobalamins were studied. These were methylcobalamin, ethylcobalamin and isopropylcobalamin (increasing substitution on $C_\alpha$); ethylcobalamin, n-propylcobalamin, isobutylcobalamin and neopentylcobalamin (increasing substitution on $C_\beta$); cyclopropylcobalamin, cyclobutylcobalamin, cyclopentylcobalamin and cyclohexylcobalamin (increasing ring size).

2) To find an alkylcobalamin which can serve as a model for the cleavage of the adenosylcobalamin Co-C bond during the isomerase enzyme reactions. This involves finding an alkylcobalamin which undergoes homolytic fission at room

Table 1.2 - Experimental Data on the Adenosylcobalamin-dependent Rearrangement Reactions

| Experimental data | Enzymes | References |
|---|---|---|
| Detection of $B_{12r}$ and organic free radicals using UV-visible and ESR spectroscopy | Dioldehydrase<br>Glycerol dehydrase<br>Ethanolamine ammonia lyase | 14, 17<br>14, 18, 19<br>14 |
| No exchange of hydrogen with solvent water | All | 7, 14-16 |
| The migrating hydrogen atom becomes equivalent to the two hydrogen atoms of C-5' of adenosylcobalamin | Dioldehydrase<br>Glutamate mutase<br>Methylmalonyl-coenzyme A mutase | 14, 15, 17<br>14<br>14, 20 |
| Formation of 5'-deoxy-adenosine as an intermediate | Dioldehydrase<br>Ethanolamine ammonia lyase<br>L-β-lysine mutase<br>Methylmalonyl-coenzyme A mutase | 14, 15, 17<br>14<br>14<br>14 |

temperature and where the Co-C bond is labilized by steric distortion.

3) To find a model reaction for the rearrangement step of the isomerase enzyme reactions, specifically in order to test whether or not isomerization of the enzyme substrates is likely to occur while the substrate is coordinated to the cobalt atom. This involves preparing an alkylcobalamin containing an alkyl group which is known to undergo rearrangement as a free radical and observing whether the rearrangement of the alkyl group is speeded up or slowed down by attachment to the cobalt.

The experimental work reported in this thesis can be divided into three parts:-

1) Preparation of organocorrinoids (Chapter 3).

2) Spectra and equilibria of organocorrinoids (Chapters 4 and 5).

3) Reactions of organocorrinoids (Chapters 6 - 9).

The first part describes the preparation of organocorrinoids; some are new and some have been prepared before. In the second part the spectra and equilibria of organocobalamins are studied in detail. The trends in the spectra and equilibria of alkylcobalamins in the series given above are shown to depend on the degree of steric interaction of the organoligand with the rest of the cobalamin molecule. Chapter 6 describes the homolytic fission of neopentylcobalamin, which is considered as a possible model for the homolytic cleavage of the Co-C bond of

adenosylcobalamin during the enzyme catalysed isomerization reactions. In Chapter 7 the reactions of organocobalamins, viz. β-elimination and homolytic fission, are described and considered in terms of the steric effects of the organoligands. In Chapter 8 the isomerization of cyclopropylmethylcobalamin to 3-butenylcobalamin is described and it is concluded that the reaction involves free-radical intermediates and does not occur while the cyclopropylmethyl group is attached to cobalt. Chapter 9 uses the results of the previous chapters to illuminate two steps in the mechanism of the isomerase enzyme reactions viz. the homolytic fission of adenosylcobalamin and the rearrangement of the substrate. Chapter 10 summarizes the main results and conclusions.

## 1.9 Literature survey

In 1975, when this work was begun, very little was known about the steric effects of organoligands. Many primary organocobalamins had been prepared[5c] but only two secondary ones viz. isopropylcobalamin[21] and cyclohexycobalamin.[22] Isopropylcobalamin and cyclohexylcobalamin have UV-visible spectra in neutral solution similar to those of the corresponding cobinamides[21,23] and it was proposed that they exist mainly in a form where the benzimidazole base is not coordinated to the cobalt.[20,21] Isopropylcobalamin and cyclohexylcobalamin spontaneously decompose in neutral solution[21,22] but cyclohexylcobalamin was shown to be stabilized by protonation and

removal from coordination of the benzimidazole base.[22] The corresponding cobinamides are stable.[21,23] Brodie[23] proposed that the addition of a strong ligand to the lower coordination site labilizes cyclohexylcobinamide by forcing the cobalt atom to move into the plane of the corrin ring giving rise to increased steric hindrance.

Grate and Schrauzer in 1979[24] published a paper in which they considered alkylcobalamins similar to those described in section 1.8. They proposed a new mechanism, β-elimination, for the decomposition of alkylcobalamins and showed that increased reactivity of organocobalamins could be correlated with increased steric interactions of the organoligand. They observed that secondary and higher primary alkylcobalamins are more stable in acidic than in neutral solution and suggested that the removal of the benzimidazole base from coordination stabilizes the organocobalamin by a conformational change of the corrin ligand. Neopentylcobalamin has been prepared and shown to be stable under anaerobic conditions but decomposes in neutral solution in the presence of air and isopropanol.[25]

Model reactions,[20,26-34] (in the absence of protein) have been found for two of the three enzymatic carbon-skeleton rearrangements ie. α-methyleneglutarate mutase and methylmalonyl-coenzyme A mutase. These model rearrangements occur during photolysis or thermolysis of organocobalamins or organocobaloximes (cobalt complexes with the bis dimethylglyoxime ligand) having ligands with the same carbon skeleton as the enzyme substrates.

# CHAPTER 2 - MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 *Cobalamins and cobinamides*

Samples of $B_{12}$ and $B_{12a}$ were supplied by Mr A P Domleo of Glaxo-Allenbury (Pty) Limited and a sample of adenosylcobalamin by Dr L Mervyn of Glaxo Laboratories, England. Methylcobalamin was prepared by Dr R A Firth and methylcobinamide by Dr R G Thorp. Some of the aquocyanocobinamide used in this work was prepared by Mr E A Betterton by the method given in section 3.6.1. Other organocobalamins and organocobinamides were prepared as described in Chapter 3.

### 2.1.2 Alkyl halides

Methyl iodide (Merck ninety-nine per cent), cyclopropyl bromide (Merck for synthesis, ninety-eight per cent), cyclobutyl bromide (Fluka, purum, $\geq$ ninety-eight per cent) and neopentyl bromide (Fluka, purum, $\geq$ ninety-seven per cent) were used as received. Ethyl bromide and n-propyl iodide (both Hopkin and Williams, GPR), isopropyl bromide and cyclopentyl bromide (both Schuchardt), isobutyl bromide (BDH, laboratory reagent) and cyclohexyl bromide (Emanuel, ninety-five per cent) were all redistilled before use.

The purity of neopentyl bromide and the secondary alkyl

halides, isopropyl bromide, cyclopentyl bromide and cyclohexyl bromide (where the corresponding alkylcobalamins cannot be purified by column chromatography) was checked using gas chromatography. The purity of these alkyl halides is given in table 2.1 together with the experimental conditions used.

Table 2.1 - Retention Times and Percentage Purity of Alkyl Halides

| Alkyl halide | Retention time (minutes) | Percentage purity |
|---|---|---|
| Isopropyl bromide[a] | 1,7 | 98 |
| Neopentyl bromide[a] | 2,6 | 98 |
| Cyclopentyl bromide[b] | 3,2 | 99 |
| Cyclohexyl bromide[c] | 3,5 | 99 |
| Cyclobutyl bromide[d] | 7,0 | $\geq$ 99 |
| Cyclopropylmethyl bromide[d] | 7,5 | 97 |
| 3-Butenyl bromide[d] | 5,5 | $\geq$ 99 |

a. Column 1, temperature = 150°C, carrier gas pressure = 12psi
b. Column 1, temperature = 170°C, carrier gas pressure = 14psi
c. Column 1, temperature = 190°C, carrier gas pressure = 14psi
d. Column 2, temperature = 42° C, carrier gas pressure = 6psi

For the columns used see section 2.2.9.

Cyclopropylmethyl bromide and 3-butenyl bromide were prepared and characterized as described in Appendix 1. The purity of these alkyl halides and that of cyclobutyl bromide is given in Table 2.1, together with the experimental conditions.

2.1.3 Buffers

The buffer systems used were based on phosphate and acetate.

The phosphate buffer consisted of 0,2M potassium dihydrogen phosphate (Hopkin and Williams) and 0,2M disodium hydrogen phosphate (BDH) mixed together in the required proportions to give pH values 5,5 - 7,0.[35] The acetate buffer consisted of 0,2M sodium acetate (BDH) and 0,2M acetic acid (Protea) mixed together in the required proportions to give pH values 3,7 - 5,6.[35]

2.1.4 Other reagents

The materials used in the preparative work were sodium borohydride, cobalt nitrate (both Hopkin and Williams), ceric nitrate (Schuchardt), phenol (Merck 'for analysis'), chloroform, diethyl ether (both Hopkin and Williams 'Analar'), sec butanol (BDH 'laboratory reagent') and glacial acetic acid (Protea).

For other work, the following reagents were used as received: methanol, n-propanol, isopropanol, dimethylformamide, cysteine hydrochloride (all Merck $\geq$ ninety-nine per cent), thioglycollic acid (BDH 'laboratory reagent', ninety-seven per cent), neopentane (Matheson, CP). Imidazole (Aldrich ninety-nine per cent) was decolourized using charcoal and recrystallized three times from benzene; pinacol (BDH 'laboratory reagent') was recrystallized from diethyl ether.

2.2 Methods

A description of the general application and the theoretical principles of the various methods used is given in this chapter. Specific applications of these methods are given in the appropriate chapter. All of these methods have been used previously but they have been adapted to fit the requirements of this work.

2.2.1 Phenol-chloroform extraction[36,37]

Because of their large number of amide side-chains, which can participate in hydrogen bonding with phenol, corrinoids are soluble in mixtures of phenol and chloroform and phenol-chloroform extraction can be used to purify them.[36] The aqueous solution of the corrinoid is extracted with portions of 1:2 (weight:volume) phenol-chloroform. The corrinoids dissolve in the phenol-chloroform phase, while the inorganic impurities remain in the aqueous phase. The phenol-chloroform extract is washed twice with water. The corrinoids are then displaced back into water by adding two volumes of ether. Organic impurities are left behind in the organic phase. The aqueous extract is then washed twice with ether to remove traces of phenol and chloroform.

2.2.2 Column chromatography [37,38,39]

The ion-exchange celluloses used were carboxymethyl (CM) and diethylaminoethyl (DE) cellulose.[40] CM cellulose contains negatively charged groups $R\text{-}O\text{-}CH_2COO^-$ (R = cellulose) and is a weakly acidic cation exchanger. DE cellulose contains positively charged groups $R\text{-}O\text{-}CH_2CH_2\text{-}\overset{+}{N}HEt_2$ and is a weakly basic anion exchanger. The grades of ion-exchangers used were CM 32 and DE 32 (Whatman) which are microgranular forms of ion-exchange cellulose. The use of a microgranular grade of ion-exchange cellulose gave very efficient separation of corrinoids, but the flow rate of eluant through the column was rather slow.

Ion-exchange celluloses are prepared as follows:-[40]

For activation of the ion-exchange cellulose twenty grams of cellulose is stirred in fifteen volumes of 'first treatment' acid or base (0,5M sodium hydroxide for CM, 0,5M hydrochloric acid for DE) and left for thirty minutes. The supernatant is filtered off and the cellulose washed in a Buchner funnel until the effluent is at the 'intermediate pH' (pH = 8 for CM, pH = 4 for DE). The ion-exchanger is then stirred into fifteen volumes of 'second treatment' acid or base (0,5M hydrochloric acid for CM, 0,5M sodium hydroxide for DE) and left for thirty minutes. The supernatant is again filtered off and the cellulose washed in a Buchner funnel until the effluent is neutral. Activation of CM and DE ion-exchange cellulose is necessary because these celluloses are supplied in the hydrogen and free base forms respectively.

The ion-exchanger is dispersed in water to give a slurry of thirty ml/g dry ion-exchanger. The slurry is gently stirred and allowed to stand twenty minutes, after which the 'fines' (small fibres of cellulose which remain in suspension) are removed by pouring off the water. Water (twenty per cent of the wet settled volume of the cellulose) is then added to the cellulose. The 'fines', if present when the cellulose is packed in the column, slow down the rate of flow of the column.

Columns are packed by pouring the slurry into the top of the column and allowing the water to drain off at the bottom. The columns used were glass columns with a plug of glass wool at

the bottom. The cellulose is allowed to settle under the influence of gravity and the flow of water. The dimensions of the columns used were 1cm x 10cm or 2,5cm x 30cm (for large-scale preparations).

Concentrated solutions were applied to the top of the column using a syringe or Pasteur pipette. The eluting solvent was deionized water. Elution using water is slow for CM cellulose and was sometimes speeded up by substituting $10^{-3}$M perchloric acid for water as the eluting solvent. Attempts to speed up elution by passing water through the column under pressure were unsuccessful; the rate of flow was increased but the efficiency of the separation was correspondingly decreased.

Separation of corrinoids on ion-exchange cellulose depends both on the overall charge on the molecules and on specific charges at local sites. $B_{12a}$ which, on elution by water, has a positive charge is not retarded by DE cellulose (an anion exchanger) but is retained by CM cellulose (a cation exchanger). Organocobalamins, which are uncharged when water is used as the eluant, are not retained by DE cellulose but are selectively retained by CM cellulose. The rate of elution from CM cellulose depends qualitatively on the pKa of the organocobalamin, organocobalamins with a high pKa being eluted more slowly, and CM cellulose can therefore be used to separate organocobalamins from each other.[38]

Organocobinamides, aquocyanocobinamide and

hydroxoaquocobinamide (overall charge + 1) on elution with water are all passed through DE cellulose but are retained to varying degrees on CM cellulose. Dicyanocobinamide is retained by DE cellulose. Hydrolysis products of organocobinamides, usually having zero overall charge, are retained by DE cellulose, presumably due to specific interactions with the carboxylate groups.[39]

2.2.3 Thin-layer chromatography[38,39,41]

Thin-layer chromatography (TLC) was done on cellulose-coated TLC plates (Merck, cellulose precoated, layer thickness 0,1mm, 20 x 20m) using solvent systems based on sec-butanol-water. The plates were cut smaller if necessary, to give dimensions of 5 x 20cm or 10 x 20cm.

The three different solvent systems used were:-[38,39,41]

(I sec-butanol/water 95:40

(II sec-butanol/glacial acetic acid/water 100:1:50

(III sec-butanol/0,88 ammonia/water 95:6,75:40.

The TLC plates were developed in glass tanks (approximately 25 x 25 x 5cm) which were lined with filter paper and which contained 150ml of solvent system I, II or III. The closed tanks containing the solvent were allowed to stand at least one hour before the TLC plates were put in. This was done in order to saturate the vapour in the tank with solvent vapour. Concentrated aqueous solutions of the corrinoid sample were applied, using a microsyringe, to the TLC plate and the spots

dried using compressed air or an electric hairdrier. The plates were developed for four hours.

The corrinoids are very intensely coloured and can easily be detected visually. Some organocorrinoids gave yellow spots which were not readily detected on the white plates but these spots could be photolysed to give red $B_{12a}$ or diaquocobinamide. This technique is very sensitive and impurities of the order of one per cent of the total sample are easily detected.

For thin-layer chromatography on cellulose $R_f$ values (distance travelled by sample/distance travelled by solvent front) are very sensitive to small variations in experimental conditions eg. temperature, development time, solvent composition and thickness of the cellulose coating. Because of this the $R_f$ value of $B_{12}$ is used as a standard in TLC.[38,39,41] $B_{12}$ was spotted either next to the corrinoid spot or on the same position as the corrinoid spot. All results for TLC were quoted as $R_{B12}$ values ($R_f$ of corrinoid/$R_f$ of cyanocobalamin), which are much more reproducible than $R_f$ values.

Thin layer chromatography is very sensitive to changes in the organoligand which makes it useful as a technique for identifying particular organocorrinoids. The $R_{B12}$ values for cobinamides are similar to those for the corresponding cobalamins. It appears that the most important factor in determining the $R_{B12}$ value of an organocorrinoid is the ability of the organoligand to participate in hydrogen bonding. The $R_{B12}$ value is low if

the ligand can form strong hydrogen bonds (eg.$HOCH_2CH_2$-) and high if it cannot (eg. $CH_3$-, $CH_3CH_2$-).[41]

2.2.4 Rotary evaporation and freeze-drying[38,39]

The rotary evaporator used was a Büchi Rotovapor-RE. Water was removed at about 50°C using a water-pump. The freeze-drying apparatus consisted of four round-bottomed flasks connected to a vacuum pump via two liquid nitrogen traps. The solution of the corrinoid to be freeze-dried was frozen in liquid nitrogen before freeze-drying was begun. Freeze-drying gave non-crystalline, very dry solid corrinoids.

2.2.5 Ultraviolet-visible spectra and extinction coefficients

Ultraviolet (UV)-visible absorption spectra were recorded over the wavelength range 300-650nm using the following spectrophotometers:- Unicam SP 8000, Unicam SP 1800 and Jasco Uvidec-1. The spectrophotometers were calibrated using holmium glass as a standard. Spectra were usually recorded with quartz cells of pathlength 10mm. Occasionally cells of pathlength 1, 40, 50 or 100mm were used. The Unicam SP 8000 spectrophotometer was useful for following the course of a reaction over a range of wavelengths and for determining the positions of isobestic points since it could instrumentally superimpose a series of spectra on the same sheet of chart-paper. The Jasco spectrophotometer has a digital read-out and was particularly suited to work where absorbance readings were taken at specific wavelengths. The Unicam and Jasco spectrophotometers had

temperature control units and the cell temperature could be varied varied from 0°C to 100°C.

Molar extinction coefficients of corrinoids[38,39] cannot be determined by measuring the absorbance of solutions of a known weight of corrinoid because corrinoids (as solid samples) contain a large and variable amount of water. The molar extinction coefficient of dicyanocobalamin has been determined with reference to a sample of $B_{12}$ of known molecular weight, including associated water, (from X-ray analysis).[42] Since most cobalamins can be quantitatively converted to dicyanocobalamin in solution their extinction coefficients can be determined with reference to that of dicyanocobalamin ( $\varepsilon = 3,04 \times 10^4$ for the γ-band at 368nm). The same value is used for the corresponding band in dicyanocobinamide since the positions and relative intensities for the peaks above 300nm of dicyanocobalamin and dicyanocobinamide are the same.[39]

Corrinoids, other than organocorrinoids, were converted to the dicyanide by adding a small crystal of potassium cyanide to the solution. Organocorrinoids were converted to the dicyanide form, after the addition of potassium cyanide, by photolysis.

2.2.6 Measurement of pH and determination of pKa values

The pH of solutions was measured using a Metrohm digital E532 pH meter. The electrodes used were a Metrohm glass electrode and a Metrohm glass microelectrode (specially for the reading of pH in spectrophotometer cells). The reference electrolyte was 3M potassium chloride. The pH meter was

standardized using BDH phosphate buffer solution pH 7,0 $\pm$ 0,01 at 20°C.

For the determination of pKa values[43] organocobalamins were dissolved in dilute sulphuric acid in a spectrophotometer cell to give pH = 1. The organocobalamin was then titrated with 0,1 - 1,0M sodium hydroxide solution using a microsyringe. The absorbance was measured spectroscopically at one or two wavelengths or, in some cases, the absorption spectrum from 300-650nm was run. The pH was measured using the glass microelectrode described above.

2.2.7 Photolysis

Tungsten lamps of 60W and 200W were used. The sample was placed at a distance of 15cm unless otherwise specified. Photolysis was generally used for the determination of extinction coefficients and for completing (to give 100 per cent product) slow thermal reactions of organocobalamins (60W lamp). The effect of photolysis on the conversion of cyclopropylmethylcobalamin to 3-butenylcobalamin was also determined (200W lamp).

2.2.8 Deoxygenation of solutions

Experiments involving small volumes of solution (2-3ml) were done in spectrophotometer cells which were closed using a rubber septum (Suba-Seal). These solutions were deoxygenated by passing a stream of oxygen-free nitrogen through the olution for five minutes. For experiments involving larger volumes of solution ($\geq$ 200ml) the solutions were degassed under vacuum for

twenty minutes, followed by a brisk stream of oxygen-free nitrogen for twenty minutes.

2.2.9 Gas chromatography

The instruments used were a Packard model 427 gas chromatograph and a Pye series 105 gas chromatograph, both of which had a flame ionization detector. The Packard gas chromatograph used helium as the carrier gas and the Pye gas chromatograph used nitrogen as the carrier gas. The columns used were

1) a 1,5m x 4mm column of 5 per cent GE-SE 52 on Anakrom and
2) a 1,5m x 6mm column of five per cent GE-SE 52 on Supelco.

Column 1 was used with the Packard gas chromatograph and column 2 with the Pye gas chromatograph.

Gas chromatography was used for determining the purity of alkyl halides used in the preparation of organocorrinoids. The retention times and percentage purity of alkyl halides are given in Table 2.1, together with the experimental conditions. Gas chromatography of the vapour phase over reaction mixtures containing neopentylcobalamin was used to detect the presence of neopentane. For this the Packard gas chromatograph and column 1 were used.

# CHAPTER 3 - PREPARATION AND PURIFICATION OF ORGANOCORRINOIDS

## 3.1 General features of the preparation, purification and identification of organocorrinoids

### 3.1.1 Preparation[5c,9]

Organocorrinoids are usually prepared by first reducing a cobalt (III) corrinoid (eg. $B_{12}$, $B_{12a}$, diaquocobinamide, aquocyanocobinamide) to a cobalt (I) corrinoid, followed by reaction of the cobalt (I) corrinoid with the required alkylating agent. Cobalt (III) corrinoids can be reduced using reducing agents such as sodium borohydride, zinc in the presence of ammonium chloride or chromium (II) in alkaline solution or by controlled potential reduction. Reduction of cobalt (III) corrinoids to cobalt (I) corrinoids, always proceeds via cobalt (II) corrinoids. Cobalt (I) corrinoids are extremely strong nucleophiles and take part in rapid addition and substitution reactions with a large variety of electrophiles. Alkylating agents commonly used are alkyl halides and tosylates, epoxides, alkenes (with electron-withdrawing substituents) and alkynes. The reaction of cobalt (I) corrinoids with alkyl halides usually proceeds via a $S_{N2}$ mechanism[44,45] but for bulky alkyl halides a mechanism involving electron transfer from cobalt (I) to the alkyl halide giving cobalt (II) and an alkyl radical followed by combination of cobalt (II) and the alkyl radical to give an alkylcobalamin may be operative.[46]

Preparations involving cobalt (I) corrinoids are very versatile. The variety of organocorrinoids which can be prepared using cobalt (I) corrinoids is limited only by steric hindrance of the alkyl group which gives rise either to very slow reactions or to unstable organocorrinoids which cannot be isolated or purified.

In this work all preparations of organocobalamins and organocobinamides were done using $B_{12a}$ or aquocyanocobinamide as the starting materials. The reducing agent was sodium borohydride and the alkylating agents used were alkyl halides (section 2.1.2). The following equations describe the reaction sequence used:-

$$Co(III)\text{-}OH_2 \xrightarrow{NaBH_4} Co(I) \xrightarrow{R\text{-}Hal} Co(III)\text{-}R$$

3.1.2 Purification

The purification of organocorrinoids requires:-

1) separation of corrinoids from excess inorganic (eg. sodium borohydride, metal salts) and organic (eg. alkyl halides) reagents.

2) separation of the required organocorrinoid from other corrinoids present, eg. unreacted starting material, corrinoids with hydrolysed side-chains.

Separation of organocorrinoids from excess organic and inorganic reagents is usually accomplished using phenol-chloroform extraction (section 2.2.1). For the separation of the required corrinoid from other corrinoids present, ion-exchange

chromatography on cellulose can be used (section 2.2.2). The purity of corrinoids can be determined by thin-layer chromatography (section 2.2.3).

All steps in the preparation and purification of organocorrinoids must be carried out in the dark or under subdued red light as organocorrinoids are light-sensitive.

3.1.3 Identification[5e]

Because the organic ligand is a very small part of the corrin molecule (in methylcobalamin for example the molecular weight of the organoligand is fifteen compared to a total molecular weight of about 1 800) most conventional techniques are of little use in determining the composition of organocorrinoids. Microanalysis is unreliable both because of the smallness of the variation in the molecular formula with change of organoligand and because of interference from the cobalt and phosphorous atoms.[37] Techniques which rely on comparison with authentic samples eg. NMR and IR spectra have been of little use because signals due to the organoligand are swamped by signals from the corrin ligand. The usefulness of NMR in the determination of the structure of organocorrinoids has increased in the last few years with better resolution of NMR spectra and the introduction of time-averaging techniques, but it is still not possible to routinely apply NMR to all organocorrinoids. The structure of naturally occurring adenosylcobalamin has been determined using X-ray crystallography[47] and synthetic adenosylcobalamin has been

shown to be identical to naturally occurring adenosylcobalamin in its chromatographic, electrophoretic and UV-visible spectral properties.[37] The structure of synthetic adenosylcobalamin is therefore assumed to be the same as that of naturally occurring adenosylcobalamin. For other, newly-prepared, organocorrinoids the organocorrinoid is shown to be pure using chromatographic techniques and its structure then inferred by analogy to adenosylcobalamin and other known organocorrinoids. All simple alkylcorrinoids exhibit UV-visible spectra which are similar to that of adenosylcobalamin in either the protonated 'base-off' or 'base-on' form and this type of spectrum is therefore diagnostic of organocorrinoids; however each alkylcobalamin or alkylcobinamide has a slightly different UV-visible spectrum which can be used in its identification.

The structure of a newly prepared organocorrinoid is inferred by reference to

1) the structure of the alkylating agent used

2) the identity of the electronic spectrum and chromatographic properties to those of an authentic sample (where available).

The presence of the Co-C bond is confirmed using the following reactions, which are typical of organocorrinoids,

1) decomposition in the presence of light and air to give aquocorrinoids [5f]

2) decomposition in the presence of light, air and cyanide to give cyanocorrinoids [5f]

3) the pH-dependent equilibrium between the red 'base-on' form and the yellow protonated 'base-off' form (for organocobalamins only).[5g]

3.2 Reduction of $B_{12a}$ and aquocyanocobinamide to $B_{12s}$ and $B_{12s}$(cobin)[5h,9]

$B_{12a}$ and aquocyanocobinamide are reduced by sodium borohydride at room temperature in neutral or alkaline solution under nitrogen to $B_{12s}$ or $B_{12s}$(cobin) via the corresponding Co(II) corrinoid. It was found that reduction proceeded rapidly to give the Co(II) corrinoid but reduction to Co(I) corrinoid was slow and irreproducible. The reduction of $B_{12}$ and $B_{12a}$ to $B_{12s}$ by borohydride is catalysed by the addition of transition metal ions.[9,48]

In order to see which metal ions are capable of catalysing the borohydride reduction, metal salts were added to a solution of $B_{12a}$ followed by addition of sodium borohydride. In all cases the concentration of $B_{12a}$ was $2,7 \times 10^{-5}$M, the concentration of sodium borohydride was $9,3 \times 10^{-2}$M and the metal ion concentration was $1,4 \times 10^{-4}$M. The following metal ion salts were added: $VOSO_4 . 5H_2O$, $[CrCl_2(H_2O)_4]Cl$, $MnSO_4$, $FeSO_4$, $Co(NO_3)_2$, $Ni(SO_4)$, $Cu(SO_4)$, $ZnSO_4$, $Na_2MoO_4$. The time taken to give complete formation of $B_{12s}$ (as shown by its UV-visible spectrum) was decreased from seven minutes (in the absence of any added metal ions) to two - three minutes by the addition of V(IV), Co(II), Ni(II) and Cu(II) ions. The others had no effect.

The addition of these metal ions leads to the formation of transition metal borides[49] (sometimes seen as black particles in suspension) which catalyse the reduction of Co(II) corrinoids to Co(I) corrinoids by borohydride. In some organocorrinoid preparations, where the rate of reduction of cobalt (III) corrinoids was irreproducible, cobalt nitrate was routinely added to the solution. The irreproducibility presumably arises from varying concentrations of trace metals in the different samples of $B_{12a}$, aquocyanocobinamide, sodium borohydride and 'deionized' water.

## 3.3 Preparation of stable organocobalamins[5c,9,37]

The following organocobalamins were prepared by the method given below: ethylcobalamin, n-propylcobalamin, cyclopropylcobalamin, isobutylcobalamin, cyclobutylcobalamin, cyclopropylmethylcobalamin, 3-butenylcobalamin. None of these alkylcobalamins except ethylcobalamin and n-propylcobalamin had been prepared before the start of this work. The methylcobalamin used was prepared by R A Firth more than ten years ago. UV-visible spectroscopy and thin-layer chromatography showed that the methylcobalamin was ~ ninety-nine per cent pure ie. no significant decomposition had occurred in ten years. The method of preparation is exemplified by the preparation of cyclobutylcobalamin.

### 3.3.1 Cyclobutylcobalamin

$B_{12a}$ (0,5g, $3,7 \times 10^{-4}$ mole) in 25ml deionized water was reduced under nitrogen using sodium borohydride (0,6g, 0,016 mole),

giving the greyish-green $B_{12s}$. The flow of nitrogen was stopped and cyclobutyl bromide (0,5ml, $3,7 \times 10^{-3}$ mole) was added to the $B_{12s}$ solution and the solution allowed to stand one hour. After this time the solution was red in colour, indicating the formation of cyclobutylcobalamin. The solution was extracted through phenol-chloroform (section 2.2.1) to remove unreacted alkyl bromide and inorganic ions and then chromatographed on CM ion-exchange cellulose (section 2.2.2) using water as the eluant. A small amount of $B_{12a}$ was retained at the top of the column and the cyclobutylcobalamin was slowly eluted from the column. The eluate from the column was again extracted through phenol-chloroform in order to concentrate it and then freeze-dried to give a red solid. The yield of cyclobutylcobalamin was eighty per cent.

This preparation could be scaled down five-fold so that 0,1g of $B_{12a}$ was used.

Thin-layer chromatography showed the presence of less than one per cent impurity. The UV-visible spectrum was that expected for an organocobalamin and the expected pH-dependent equilibrium between the red 'base-on' and yellow protonated 'base-off' forms was observed. Decomposition in the presence of light to give $B_{12a}$ and in the presence of light and cyanide to give dicyanocobalamin occurred as expected, showing the presence of the Co-C bond.

3.3.2 *Other organocobalamins*

The above procedure was used for the preparation of other

organocobalamins. The yield of organocobalamin was seventy to eighty per cent except in the case of cyclopropylmethylcobalamin where the yield was five to ten per cent because 3-butenylcobalamin was formed during the preparation (see section 8.3). Cyclopropylmethylcobalamin was well separated from 3-butenylcobalamin (and a trace of cyclobutylcobalamin) on a CM cellulose column, the three bands being eluted in the order 3-butenylcobalamin, cyclopropylmethylcobalamin, cyclobutylcobalamin.

The purity of the above organocobalamins was checked using thin-layer chromatography, showing $\leq$ one per cent impurity in all cases. The $R_{B12}$ values for the organocobalamins are given in Table 3.1. In all cases the spectra were as expected for organocobalamins, the expected pH-dependent equilibrium was observed, and the expected reactions in the presence of light and cyanide were observed.

All the organocobalamins were stored in containers which excluded light and organocobalamins which underwent decomposition subsequent to purification (cyclopropylmethylcobalamin and 3-butenylcobalamin) were stored at liquid nitrogen temperature.

## 3.4 Preparation of unstable secondary organocobalamins

It is well known that, while primary organocobalamins are usually stable to isolation and purification, secondary organocobalamins decompose when attempts are made to purify them from the reaction mixture.[21,22] In order to overcome this problem the organocobalamins isopropylcobalamin, cyclopentylcobalamin

Table 3.1 - $R_{B12}$ Values on Thin-layer Cellulose

| Compound | $R_{12}$ values | | |
|---|---|---|---|
| | Solvent I | Solvent II | Solvent III |
| Cobalamins | | | |
| Methyl | 1,40 | | |
| Ethyl | 1,50 | | |
| n-Propyl | 1,48 | | |
| Cyclopropyl | 1,66 | 1,51 | 1,45 |
| Isobutyl | 1,55 | 1,52 | 1,69 |
| Cyclobutyl | 1,56 | 1,58 | 1,52 |
| Cyclopropylmethyl | 1,54 | 1,54 | 1,33 |
| 3-Butenyl | 1,59 | 1,64 | 1,70 |
| Cobinamides | | | |
| Methyl | 1,40 | | |
| Ethyl | 1,49 | | |
| n-Propyl | 1,54 | | |
| Isopropyl | 1,44 | | |
| Isobutyl | 1,79 | | |
| Cyclobutyl | 1,71 | 1,90 | |
| Cyclopropylmethyl | 1,62 | 1,76 | |
| 3-Butenyl | 1,84 | 1,91 | |
| Aquocyano | 1,08 | 0,38 | |

and cyclohexylcobalamin were prepared *in situ* in a spectrophotometer cell at the pH required as follows:- $B_{12a}$ was reduced to $B_{12s}$ using sodium borohydride and alkylated using an alkyl halide. Excess sodium borohydride was destroyed using phosphate buffer pH = 5,5 (section 2.1.3) or dilute sulphuric acid.[21] The reactions were then studied spectroscopically and further reagents added as required.

The detailed procedure for the preparation of isopropyl, cyclopentyl and cyclohexylcobalamin to give final pH = 1,6 and 12 is now given. In all cases a stock solution of $B_{12a}$ ($6,0 \times 10^{-5}$M $B_{12a}$ in $2,4 \times 10^{-4}$M cobalt nitrate solution) was used.

Final pH = 1,0 Into a 1cm pathlength spectrophotometer cell was pipetted 1,0ml stock solution of $B_{12a}$ and 0,95ml deionized water. A small amount of solid sodium borohydride was added. When reduction of $B_{12a}$ to $B_{12s}$ had occurred 60µl of alkyl bromide was added, the cell closed with a Suba-Seal and nitrogen bubbled through for five minutes. When no more $B_{12s}$ was present (shown spectrophotometrically), 0,05ml of 3,6M sulphuric acid was added under nitrogen and nitrogen passed through the solution for about one minute. This gives a solution of alkylcobalamin with a concentration of $3,0 \times 10^{-5}$M at pH = 1,0.

Final pH = 6-7 Into a 1cm pathlength spectrophotometer cell was pipetted 1,0ml stock solution of $B_{12a}$ and 0,5ml deionized water. Sodium borohydride was added, followed by alkyl bromide and reduction and alkylation occurred as in the previous case. Then 0,5ml of phosphate buffer pH = 5,5

section (2.1.3) was added and nitrogen passed through the solution for one minute. This gives a solution which is $3,0 \times 10^{-5}$M in alkylcobalamin at pH = 6,3-6,6.

Final pH = 12,0. Alkylcobalamins at pH = 12 were prepared in the same way as in the previous cases except that 0,05ml water was substituted by 0,05ml 2M NaOH. The sodium hydroxide was added after the phosphate buffer. This gives a solution of alkylcobalamin at a concentration of $3,0 \times 10^{-5}$M at pH = 12,0.

The hydrogen formed in the above preparations was flushed out of the cell by a rapid stream of nitrogen.

In these preparations sodium borohydride was inactivated by the addition of either sulphuric acid or pH = 5,5 phosphate buffer. Treatment of sodium borohydride with protonic acids generates diborane which reacts with water to give boric acid.[49] The reaction of sodium borohydride with sulphuric acid is:-

$$2NaBH_4 + H_2SO_4 \longrightarrow B_2H_6 + 2H_2 + Na_2SO_4$$

$$B_2H_6 + 6H_2O \longrightarrow 2B(OH)_3 + 3H_2.$$

Sodium borohydride is hydrolysed rapidly at pH<7 but very slowly at pH>7.

Excess alkyl halide was probably present in the spectrophotometer cell for all these preparations. A brisk stream of nitrogen through the solution removed some, but probably not all, of the alkyl halide. Attempts to remove the alkyl halide by extraction with ether were unsuccessful, resulting in partial conversion of the organocobalamin to $B_{12a}$.

Another problem occasionally encountered when working with preparations of this type was turbidity due to fine particles of cobalt boride in suspension (see section 3.2). Turbid preparations were discarded. If the cobalt nitrate was omitted the reduction (and hence the alkylation) reaction became extremely slow and irreproducible.

3.5 Preparation of neopentylcobalamin

Preliminary experiments showed that neopentylcobalamin was stable in the presence of air at pH's $\leq 3$ and was stable under nitrogen at all pH's. The greater stability at acidic pH's is associated with the protonation and removal from coordination of the benzimidazole base.[23] The increased stability of organocorrinoid complexes with bulky organoligands when the benzimidazole base is removed has been observed previously. For example, the secondary organocobinamides isopropylcobinamide sec - butylcobinamide and cyclohexylcobinamide are much more stable than the corresponding organocobalamins.[21-23] Neopentylcobalamin was prepared and partially purified by doing the purification under acidic conditions, where the benzimidazole base was not coordinated and the cobalamin was stabilized.

The preparation and purification of neopentylcobalamin was performed as follows: $B_{12a}$ (0,1g, $7{,}4 \times 10^{-5}$ mole) in 10ml of a solution of $3{,}5 \times 10^{-4}$M cobalt nitrate was reduced under nitrogen using sodium borohydride (0,34g, $9{,}0 \times 10^{-3}$ mole), giving $B_{12s}$ Neopentyl bromide (70µl, $5{,}6 \times 10^{-4}$ mole) was added under nitrogen and the reaction mixture allowed to stand under nitrogen for

twenty minutes. At the end of this time the solution was orange in colour, indicating the formation of neopentylcobalamin. The sodium borohydride was inactivated and the solution made acidic by the addition of 0,2M potassium dihydrogen phosphate. The reaction mixture was then extracted through phenol - chloroform into dilute sulphuric acid, pH = 3.

Varying amounts of $B_{12a}$ (two to ten per cent as judged from the UV-visible spect. ) were found in different preparations. The $B_{12a}$ arises from incomplete reduction of starting material and/or decomposition of neopentylcobalamin during preparation and purification. Neopentylcobalamin was shown to be rapidly decomposed in the presence of a large excess of sodium borohydride ($t_{\frac{1}{2}}$ < 2 minutes in 0,5M $NaBH_4$) but under the conditions used in the preparation of neopentylcobalamin the rate of formation of neopentylcobalamin was greater than its rate of decomposition. The solution of neopentylcobalamin in dilute sulphuric acid, prepared as described above, could be stored for at least two weeks at -20°C without significant decomposition.

Neopentylcobalamin could not be purified on CM cellulose because at pH = 3 (using perchloric acid as the eluant) there is no separation of organocobalamins from each other or from $B_{12a}$ and because neopentylcobalamin shows significant decomposition at pH's ≥ 3.

Thin-layer chromatography could not be used to determine the purity of neopentylcobalamin because this organocobalamin

decomposed on the TLC plate (the solvent used was solvent III ie. sec-butanol/water/glacial acetic acid 100:1:50). A yellow-orange spot which turns red on exposure to light (neopentylcobalamin) followed by a red streak (decomposition to aquocobalamin) is seen on TLC.

Because a large excess of alkyl bromide, compared to $B_{12a}$, was used in the above preparation there was a possibility that a substantial proportion of the organocobalamin isolated was a compound other than neopentylcobalamin. Gas chromatography of neopentyl bromide shows the presence of two impurities which together represent two per cent of the total alkyl bromide. The impurities are probably isomeric pentyl bromides. In general, primary alkyl bromides should react more rapidly with $B_{12s}$ than neopentyl bromide and the resulting alkylcobalamins should be more stable than neopentylcobalamin. Secondary alkyl bromides should react more slowly with $B_{12s}$ than neopentyl bromide and the resulting alkylcobalamins should be less stable than neopentylcobalamin. It is unlikely that a secondary alkylcobalamin is present as an impurity in neopentylcobalamin but it is possible that a primary alkylcobalamin is present. In order to check whether neopentylcobalamin contained a significant proportion of organocobalamin impurity the following experiment was done:- Neopentylcobalamin was prepared as above, phenol-chloroform extracted into water instead of dilute sulphuric acid (this caused the decomposition of neopentylcobalamin to give $B_{12a}$) and

chromatographed on a CM cellulose column. Two bands were collected from the column, a fast-moving yellow band and a slow-moving red band. The fast-moving band corresponded to a primary organocobalamin (or organocobalamins) as shown by its spectrum, light-sensitivity and behaviour in the presence of acid and base. The slow-moving band was $B_{12a}$ as shown by its spectrum. The organocobalamin band corresponded to two per cent of the total cobalamin present at the start of the preparation (the recovery of cobalamin was ninety-four per cent). Neopentylcobalamin, therefore, contains two to ten per cent $B_{12a}$ and about two per cent of other organocobalamins (probably isomeric pentylcobalamins). The impurities are not present in large enough amounts to significantly affect the spectra, equilibria or reactions of neopentylcobalamin.

3.6 Preparation and purification of organocobinamides[39]

The preparation of organocobinamides from $B_{12}$ requires two steps, firstly the removal of the 5,6-dimethylbenzimidazole nucleotide, and secondly the alkylation of the nucleotide-free product.

The nucleotide can be removed from $B_{12}$ using cerous hydroxide. Cerous hydroxide hydrolysis of $B_{12}$ gives aquocyanocobinamide as the corrinoid product as well as the 5,6-dimethylbenzimidazole nucleoside and phosphate.[50] The aquocyanocobinamide can be reduced to give $B_{12s}$ (cobin) and alkylated analogously to $B_{12a}$, giving organocobinamides.

3.6.1 Aquocyanocobinamide[13]

For the preparation of aquocyanocobinamide the procedure followed was that of Thorp,[39] which was based on that of Friedrich and Bernhauer.[50]

Aquocyanocobinamide was prepared using $B_{12}$(1,0g, 6 x $10^{-4}$mole), cerous nitrate (84ml of 0,33M $Ce(NO_3)_3 \cdot 6H_2O$,) sodium hydroxide (25ml of 2M NaOH), potassium cyanide (0,84gKCN in 12,5ml water). These reagents were added to 150ml water and refluxed over a boiling water bath for thirty minutes with frequent shaking. The mixture was cooled in ice, made basic (pH = 8,5) with 0,880 ammonia and left in a refrigerator overnight to allow the cerous hydroxide to coagulate. The solution was then filtered using a sinter-glass funnel and extracted with phenol-chloroform. The product at this point was mainly dicyanocobinamide. In order to convert the dicyanide to the aquocyano form the solution was made acidic with 1M perchloric acid and shielded from the light to prevent photolysis to diaquocobinamide. A stream of nitrogen was passed through the solution (to remove hydrogen cyanide formed) for two days until most of the dicyanocobinamide had been converted to aquocyanocobinamide (shown by the spectrum of the neutralized species). The solution was neutralized using sodium hydroxide, extracted through phenol-chloroform and concentrated by rotary evaporation tc small volume. This solution was made alkaline (pH = 8) and chromatographed on columns of DE and CM cellulose, connected in series, using water as the eluant. The

aquocyanocobinamide band was not retarded by the DE cellulose but was retained at the top of the CM column. Products of further hydrolysis and small amounts of dicyanocobinamide were retained by the DE cellulose. $B_{12}$ passed through both columns. Hydroxoaquocobinamide (this was not always present) passed through both columns as a broad, slow-moving band. The two columns were disconnected once the aquocyanocobinamide had moved off the DE column and elution continued until the hydroxoaquocobinamide band was well separated from the aquocyanocobinamide band. The aquocyanocobinamide band at the top of the CM column was then removed from the column and the product eluted with $10^{-3}$M perchloric acid. The solution was again neutralized using sodium hydroxide, and rotary evaporated. The yield was 0,6g or seventy per cent.

The product was shown to be pure using thin-layer chromatography. Two spots of approximately equal intensity were observed. The two spots were due to the presence of two isomers (aquo)cyanocobinamide and (cyano)aquocobinamide.[39] For $R_{B12}$ values for aquocyanocobinamide see table 3.1.

3.6.2 Isobutylcobinamide

The preparation of isobutylcobinamide is given below as an example of the method of preparation of organocobinamides.

Aquocyanocobinamide (0,05g, 3 x $10^{-5}$ mole) in 25ml water was reduced under nitrogen (cobin). The solution turned purple due to the presence of dicyanocobinamide formed at the

same time as $B_{12r}$ (cobin) and $B_{12s}$ (cobin). To this solution was added 50µl ($3,7 \times 10^{-4}$ mole) isobutyl bromide. After one hour the yellow organocobinamide had been completely formed and the reaction mixture was extracted through phenol-chloroform (section 2.2.1) and purified on DE and CM cellulose as described for aquocyanocobinamide. The eluate from the column was again extracted through phenol-chloroform and freeze-dried to give a yellow solid. The yield of isobutylcobinamide was 0,02g or forty per cent.

The UV-visible spectrum was that expected for an alkylcobinamide and was very similar to that of the protonated 'base-off' form of isobutylcobalamin. The presence of the Co-C bond was confirmed by the expected decomposition in the presence of light to give diaquocobinamide and in the presence of light and cyanide to give dicyanocobinamide.

Thin layer chromatography immediately after preparation showed the presence of one yellow spot ($R_{B12}$ = 1,79 solvent I) and isobutylcobinamide was ≥ ninety-nine per cent pure.

On storage at 0° -4°C (in the freezer compartment of a refrigerator) the sample gradually decomposed as shown by thin layer chromatography. After five weeks an additional yellow spot of lower $R_{B12}$ ($R_{B12}$ = 1,65) was present, representing ~ twenty per cent of the total organocorrinoid. After five months a further yellow spot ($R_{B12}$ = 1,49) was present. At this stage the yellow spots were approximately equal in intensity,

each representing ~thirty per cent of the total organocorrinoid. The yellow spots of lower $R_{B12}$ were organocorrinoid spots as shown by their sensitivity to light (red diaquocobinamide was formed on exposure to sunlight). Over five months there was little or no change in the UV-visible spectrum. These spots were ascribed to hydrolysis of the side-chains of the cobinamides (section 3.6.3).

3.6.3 Other organocobinamides

The organocobinamides prepared by the above method were:- methylcobinamide, ethylcobinamide, n-propylcobinamide, isopropylcobinamide, isobutylcobinamide, cyclobutylcobinamide, cyclopropylmethylcobinamide and 3-butenylcobinamide. The yields of alkylcobinamides were variable (twenty to seventy per cent) due to the formation of hydrolysis products (see below).

The organocobinamides prepared by the above method have the UV-visible spectra expected for organocobinamides and where the organocobinamides have been previously prepared they have spectra identical to the published spectra.[21,39] The Co-C bond is present in all cases as shown by photolysis in the presence and absence of cyanide. The $R_{B12}$ values for organocobinamides are given in Table 3.1.

Thin-layer chromatography shows, however, that the organocobinamide preparations are sometimes inhomogeneous. In some cases there was more than one spot on TLC immediately after preparation and purification. For others there was only one

spot present immediately after preparation and purification but other spots appeared after a period of weeks to months similarly to isobutylcobinamide. All of the spots are light-sensitive and are therefore presumably due to organocorrinoids. Also, the UV-visible spectra show the presence of 100 per cent organocorrinoid and are similar to the spectra in acidic solution of the corresponding cobalamins. The extra spots probably represent organocobinamides with modified side-chains rather than compounds where changes have occurred in the organoligand or the corrin ring.

The most likely modification of the side-chains is hydrolysis of amide groups to give carboxylic acid groups. It is known that mild acid treatment of corrinoids gives rise to hydrolysis of some of the amide side-chains to carboxylic acid groups;[6] this could occur when the cobinamides are eluted from CM cellulose using $10^{-3}$ M perchloric acid. The decomposition of organocobinamides in the solid state is rather unexpected because hydrolysis of amides does not usually occur either in neutral solution or the solid state. However, corrinoids which have been freeze-dried pick up water from the atmosphere and it is known that cobalt (III) complexes catalyse hydrolysis of amides.[51] Perhaps the cobalt atom of cobinamides catalyses the hydrolysis of the amide side-chains. It appears that organocobinamides are much less stable in the solid state than are the corresponding organocobalamins eg. methylcobalamin kept for a period of ten

years is still chromatographically homogeneous but methylcobinamide, over the same period of time, gives three organocorrinoid spots ($R_{B12}$ = 1,40, 1,13 and 0,70) in approximately equal proportions as well as a trace of diaquocobinamide.

3.6.4 Organocobinamides prepared in solution

Some organocobinamides were prepared *in situ* in a spectrophotometer cell by the same method used to prepare secondary alkylcobalamins (see section 3.3). The organocobinamides prepared in this way were methylcobinamide, cyclobutylcobinamide and neopentylcobinamide. For methylcobinamide and cyclobutylcobinamide prepared in this way the UV-visible spectra were identical to the UV-visible spectra of methylcobinamide and cyclobutylcobinamide prepared as in sections 3.6.2 and 3.6.3

# CHAPTER 4 - THE EFFECT OF STERIC DISTORTION OF THE COBALT COORDINATION SPHERE ON THE SPECTRA AND pKa's OF ORGANOCOBALAMINS

## 4.1 Introduction

A great deal of work has been done on the inductive effect of axial ligands on the spectra and equilibrium constants of corrinoids[5i,5j] but very little work has been done on the steric effects of the axial ligands on the spectra and pKa's of the organocobalamins. This chapter describes a systematic study of the steric effects of the organoliganas of cobalamins on the spectra and equilibria of cobalamins.

### 4.1.1 Steric effects of the organoligand

In adenosylcobalamin the Co-$\hat{C}_\alpha$-$C_\beta$ bond angle is $125^{o}$[47] rather than the tetrahedral angle ($109,5^{o}$) expected for a $\sigma$ bond. This large deviation is presumably caused by the need to maximize overlap of the $sp^3$ orbital of the alkyl ligand with the relevant hybridized ($sp^3d^2$) orbital of the cobalt and to minimize non-bonded repulsion.[52] This repulsion originates from interactions between the electron pairs in the C-C and C-H bonds of the ligand and the non-bonded electron pairs of the cobalt (the $t_{2g}$ subshell) as well as the lone pairs of the nitrogen atoms coordinated to the cobalt.[52]

The Co-C bond in adenosylcobalamin is surrounded at $6,5\mathring{A}$ by the two-$CH_2$-groups of $C_{26}$ and $C_{37}$ and by the two methyl groups

($C_{46}$ and $C_{54}$), forming a square nearly 2Å above the cobalt.[47] Steric interactions between these groups and a bulky organoligand would be expected. In adenosylcobalamin the oxygen atom of the ribose ring is in contact with the corrin ring between C14 and C15 as shown by X-ray crystallography and interaction would be expected between this oxygen atom and the methylene groups ($C_{26}$ and $C_{37}$) of the acetamide side-chains and the methyl groups ($C_{46}$ and $C_{54}$).[47]

Adenosylcobalamin is the only organocobalamin for which X-ray crystallography data is available but for other simple organocobalamins such as ethylcobalamin the Co-$\hat{C}_\alpha$-$C_\beta$ bond angle is presumably also close to 125°. Thus, in organocobalamins in general, steric compression can originate from two sources - firstly, repulsive interactions between the electron pairs in the C-C and C-H bonds of the organoligand and the non-bonded electron pairs of the cobalt and the nitrogens and, secondly, repulsive interactions between the $C_\beta$ substituents of the organoligand and the side-chains of the corrin ring.

4.1.2 Selected series of alkyl ligands

The effect of steric distortion around the Co-C bond and between the $C_\beta$ substituents and the propionamide side-chains on the spectra, equilibria and reactions of organocorrinoids have been studied with three series of ligands. For series 1 (methyl <ethyl<isopropyl) there is increasing substitution on $C_\alpha$ of the alkyl ligand. Organocorrinoids having the tert-butyl group

(the end-member of this series) as a ligand have never been prepared, due to their instability.[24] One would expect the Co-$\hat{C}_\alpha$-$C_\beta$ bond angle to be progressively decreased in this series. For series 2 (ethyl≤n-propyl < isobutyl < neopentyl) there is increasing substitution on $C_\beta$ of the alkyl ligand. One would expect the Co-$\hat{C}_\alpha$-$C_\beta$ bond angle to be progressively increased in this series because of increasing steric interactions between the $C_\beta$ substituents and the propionamide side-chains of the corrin ring. For series 3 (cyclopropyl < cyclobutyl < cyclopentyl < cyclohexyl) one would expect the Co-$\hat{C}_\alpha$-$C_\beta$ bond angle to be decreased due to the increasing bond angle of the cycloalkane ring. These series of alkylcobalamins show gradual changes in the degree of repulsion either between the bonding electrons in the $C_\alpha$- $C_\beta$ and $C_\alpha$-H bonds on the one hand and the lone pairs on the cobalt and nitrogen atoms on the other (as in 1 and 3) or between substituents on $C_\beta$ and upward-projecting groups on the corrin ring (as in 2).

The adenosyl ligand was also studied for comparison with the ligands in series 1, 2 and 3. The cyclopropylmethyl and 3-butenyl ligands (Chapter 8) are included here for completeness.

Strictly speaking, it is not possible to separate steric and electronic effects in corrinoids. Changes in steric effects (due to non-bonded electrons) will be accompanied by some changes in electronic effects (in the Co-$C_\alpha$ bonded pair) due to changes in the hybridization of $C_\alpha$ as well as changes in the Co-$C_\alpha$ bond length.

A summary of the forms of alkylcobalamins and alkylcobinamides and their equilibria in solution is given in Fig.4.1. Capital letters in the text refer to Fig.4.1. Evidence for the presence of the 5-coordinate, unprotonated form C is discussed in section 5.3

The effect of steric distortion due to the organoligand on the UV-visible spectra of organocorrinoids and on the pH-dependent equilibria of organocobalamins will be discussed in this chapter. In the following chapter (Chapter 5) the pH-independent equilibria of organocobalamins will be discussed.

## 4.2 UV-visible spectra

The electronic transitions giving rise to the absorption spectra of corrinoids, above 300nm, occur mainly within the $\pi$ system of the corrin ligand. The d-d transitions are expected to be weak and are probably buried under the intense $\pi$-$\pi^*$ transitions. Charge transfer transitions are not generally observed in the spectra of corrinoids. The most important evidence for this is the similarity of the spectra of natural and synthetic metal-free corrinoids to those of cobalt (III) corrinoids.[5j] The spectra of metal-free corrinoids have all the main bands seen in the cobalt (III) corrinoids and they are of similar intensity to those of cobalt (III) corrinoids.[53] The electronic transitions in the region below 300nm are due to the benzimidazole ring and will not be considered here.[54]

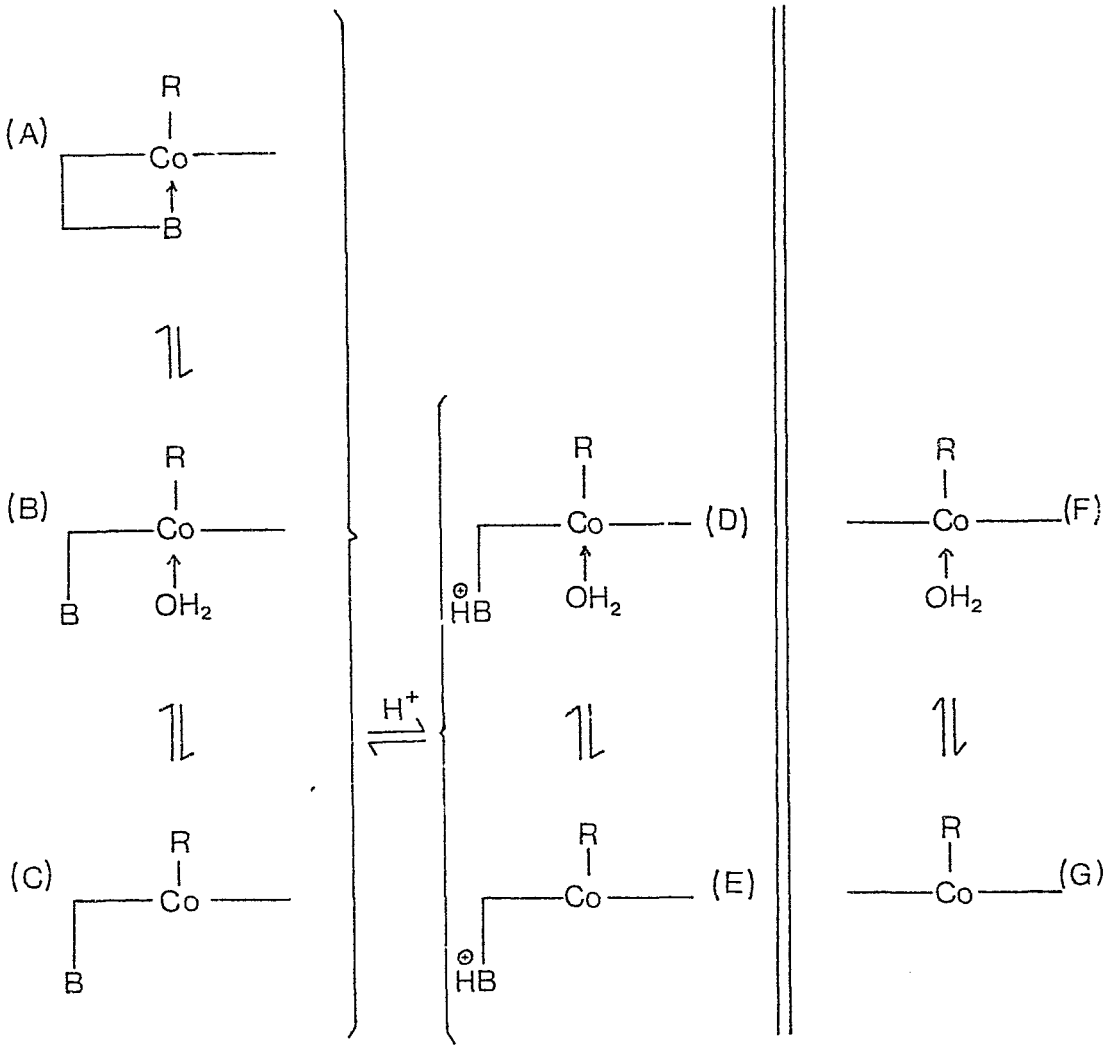



Fig.4.1. Schematic summary[5k, 21] of the equilibria involving a change in the axial ligands exhibited by organocobalamins (A-E) and organocobinamides (F, G). R is the organoligand and B is the 5,6-dimethylbenzimadazole base. C, E and G are five-coordinate complexes, the others are six-coordinate complexes. Complexes in the same vertical column are involved in pH-independent equilibria, in which increasing temperature favours the five over the six-coordinate forms.

4.2.1 Types of UV-visible spectra[5i,55]

The nomenclature of the bands in the corrinoid UV-visible spectra follows that of porphyrins.[5i]

The UV-visible spectra of corrinoids can be classified as 'normal' (eg. cyanocobalamin), 'intermediate' (eg. methylcobalamin in neutral solution) or 'anomalous' (eg. methylcobalamin in acid solution) (Fig.4.1)

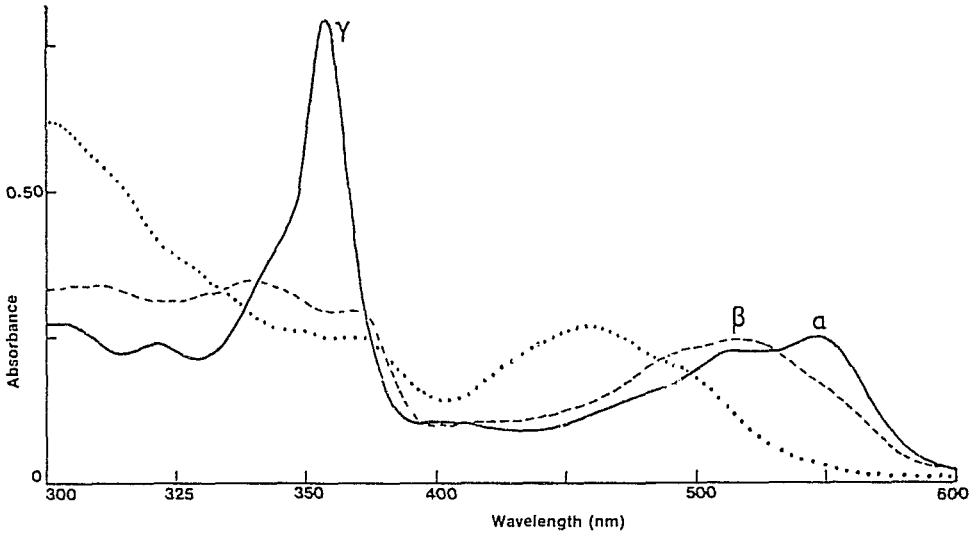



Fig.4.2 Absorption spectra of $B_{12}$(——), methylcobalamin in neutral solution (---) and methylcobalamin in acid solution (...) as examples of 'normal', 'intermediate' and 'anomalous' spectra. The α , β and γ bands are indicated for $B_{12}$

'Normal' spectra show the following bands:-

The α bands. This is a series of low to medium intensity bands in the 500nm region. The α band is the band at longer wavelength

and the β band that at shorter wavelength. The bands in this region are clearly defined.

The D and E bands. These are two bands of low intensity in the 390-420nm region.

The γ band in the 350-370nm region of the spectrum. This is the sharpest and most intense band in the spectrum.

The δ bands. Bands of low intensity in the 300-330nm region of the spectrum.

As the spectra become increasingly 'anomalous' the following changes occur:- In the αβ region intensity moves from the α to the β band with the bands becoming less clearly separated ('intermediate' spectra) and the β band moves to longer wavelength ('anomalous' spectra). Only one low-intensity band is found in the D and E region for the 'intermediate' spectra and for the 'anomalous' spectra the D and E bands are no longer seen as distinct bands. In the 300-400nm region the one γ band in the normal spectrum is replaced at first by a complex series of at least three bands extending from 300-400nm with the γ band moving out to longer wavelengths ('intermediate' spectra). All the bands are of considerably reduced intensity compared to the band found in the 'normal' spectra. In the extreme case of the 'anomalous' compounds the spectrum is again simple with a single γ band at 300-310nm.

Theoretical interpretations of the electronic transitions have been made eg.[56,57] but these will not be discussed here.

4.2.2 Spectra of protonated 'base-off' organocobalamins

All alkylcobalamins in dilute aqueous solution at pH≤ 1 exist entirely in a yellow five-coordinate (E) form where the benzimidazole side-chain is protonated and removed from coordination (see later in this section). Alkylcobalamins in acid solution have 'anomalous' spectra ie. absorption in the αβ region occurs between 440 and 460nm and bands are observed at 374-390 nm and at 303nm. The presence of only the E form at pH ≤1 is useful because the spectra at pH≤ 1 can be used to assess the effects of alkyl ligands on the properties of a single complex rather than on an equilibrium as at higher pH's. The spectra of the protonated base-off alkylcobalamins with the alkyl ligands described in section 4.1.2 were therefore studied.

The spectra were recorded at pH = 1,2 (pH = 0 for methylcobalamin and cyclopropylcobalamin) at concentrations of 3,0 or 6,0 x $10^{-5}$M. Examples of the spectra of the five-coordinate, protonated,'base-off'(E) forms are given in Figs. 4.3 and 4.4. The wavelengths and extinction coefficients of the main bands are given in Table 4.1. Beer's law plots for methylcobalamin (1,0 x $10^{-5}$M - 1,0 x $10^{-4}$M) and ethylcobalamin (2,0 x $10^{-6}$M - 2,0 x $10^{-4}$M) were linear.

Series 1 Varying the alkyl ligand in the order methyl, ethyl and isopropyl causes marked changed in the UV-visible spectrum in the 440-460nm region, in the region around 380nm and in the 303nm region. In the 440-460nm region methylcobalamin

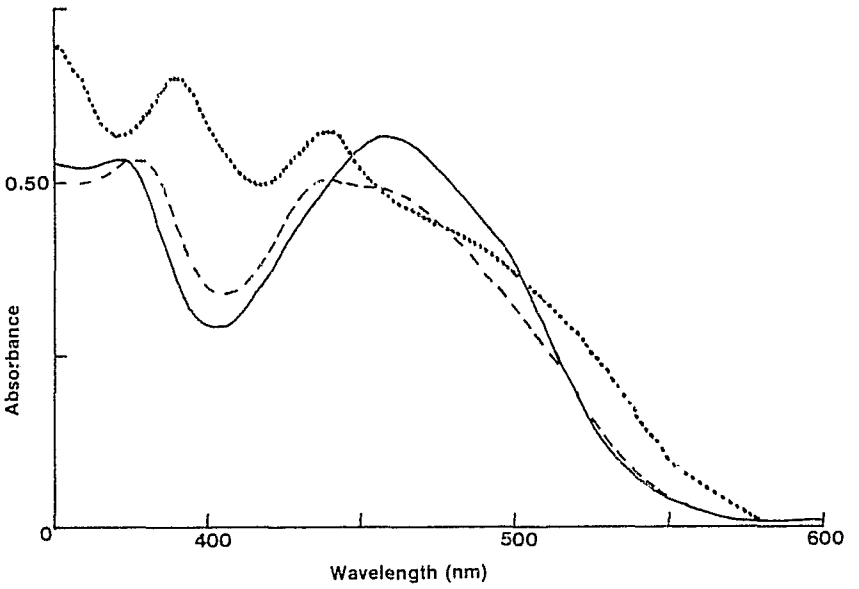



Fig.4.3 Spectra of 6,0 x $10^{-5}$M solutions of the protonated 'base-off' (E) forms of methyl (—— ), ethyl (---) and neopentyl (....) cobalamins.

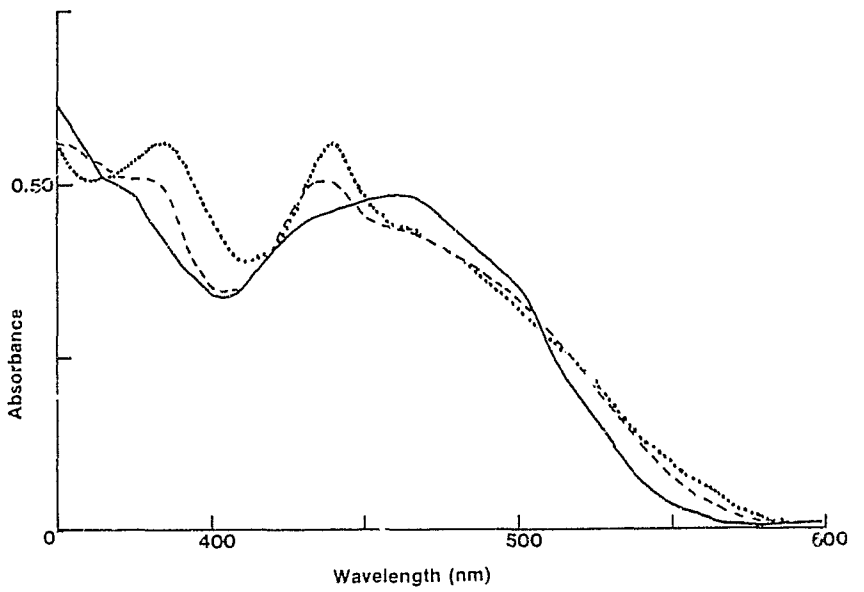



Fig.4.4 Spectra of 6,0 x $10^{-5}$M solutions of the protonated 'base-off' (E) forms of cyclopropyl (—— ), cyclobutyl (---) and cyclohexyl (....) cobalamins.

Table 4.1 - UV-visible Spectra of Protonated, 'base off' (E) forms of organocobalamins

| Ligand | αβ region Maxima | | Ratio | γ region | 330nm band |
|---|---|---|---|---|---|
| | λ /nm ($10^{-4}\varepsilon$) | | ε440:ε460 | λ/nm ($10^{-4}\varepsilon$) | $10^{-4}\varepsilon$ |
| adenosyl | 458 (0,88) | | 0,89 | 376 (0,82) | |
| methyl | 460 (0,93) | | 0,90 | 374 (0,90) | 2,2 |
| ethyl | 455 (0,82) | 440 (0,84) | 1,08 | 380 (0,91) | 3,0 |
| isopropyl | | 442 (1,1) | 1,23 | 382 (1,2) | > 3,2 |
| ethyl | 455 (0,82) | 440 (0,84) | 1,08 | 380 (0,91) | 3,0 |
| n-propyl | 455 (0,86) | 441 (0,88) | 1,05 | 381 (0,94) | 2,8 |
| isobutyl | | 439 (0,85) | 1,12 | 384,5 (0,92) | 2,9 |
| neopentyl | | 438 (0,93) | 1,22 | 388,5 (1,0) | (3,0) |
| cyclopropyl | 460 (0,83) | | 0,97 | 374 (0,83) | 2,0 |
| cyclobutyl | | 438 (0,84) | 1,19 | 378 (0,86) | 2,7 |
| cyclopentyl | | 440 (0,83) | > 1,1[a] | 382 (0,90) | 3,1 |
| cyclohexyl | | 442 (0,93) | 1,27 | 384 (0,93) | > 3,3 |
| 3-butenyl | 455 (0,78) | 440 (0,76) | 0,98 | 378 (0,88) | 2,4 |
| cyclopropylmethyl | 455 (0,79) | 442 (0,83) | 1,06 | 382 (0,99) | 2,7 |

[a] Only a minimum value could be obtained due to the presence of some $B_{12r}$ which absorbs at 470nm.

has a maximum at 460nm, ethylcobalamin has bands of near equal intensity at 440 and 455nm and isopropylcobalamin has a band at 460nm. The ratio of the extinction coefficients at 440 and 460nm - $\varepsilon$440: $\varepsilon$460 - increases in the order methyl < ethyl < isopropyl. In the region around 380nm the band at 374nm in methylcobalamin moves to higher wavelengths in the order methyl < ethyl < isopropyl. Over the series there is a rise in the intensity of the band at 303nm in the order methyl < ethyl < isopropyl.

Series 2 Varying the alkyl ligand in the order ethyl, n-propyl, isobutyl and neopentyl causes marked changes in the UV-visible spectrum in the 440-460nm region and in the region around 380nm. In the 440-460nm region all the members of this series have an absorption maximum at 440nm but for ethyl and n-propylcobalamin the absorbance at 440 and 455nm is almost equal while for isobutyl and neopentylcobalamin the absorbance at 440nm increases relative to that at 460nm. The ratio $\varepsilon$440 :$\varepsilon$460 increases in the order ethyl ~ n-propyl < isobutyl < neopentyl. In the region around 380nm the band at 380nm in ethylcobalamin moves to higher wavelengths in the order ethyl ~ n-propyl < isobutyl < neopentyl. There appears to be no significant change in intensity in the band at 303nm in this series.

Series 3 Varying the alkyl ligand in the order cyclopropyl, cyclobutyl, cyclopentyl and cyclohexyl causes changes in the UV-visible spectrum in the region around 440-460nm, in the 380nm region and in the 303nm region. In the 440-460nm region

cyclopropylcobalamin has a maximum at 460nm while the others of the series have their maxima at about 440nm. The ratio ε440 :ε460 increases over the series in the order cyclopropyl < cyclobutyl < cyclopentyl < cyclohexyl. In the region around 380nm the band at 374nm in cyclopropylcobalamin gradually shifts to higher wavelengths in the order cyclopropyl < cyclobutyl < cyclopentyl < cyclohexyl. Over the series there is a rise in the intensity of the band at 303nm in the order cyclopropyl < cyclobutyl < cyclopentyl < cyclohexyl. Comparing the spectra of cyclopropylcobalamin to isopropylcobalamin and cyclopropylmethylcobalamin to isobutylcobalamin it can be seen, as expected, that the change from a cyclopropyl to an isopropyl substituent in the $\beta$-position has very little effect on the UV-visible spectrum but the same change at the α-position has a large effect.

It is possible, looking at the 440-460nm region, to divide the alkylcobalamin spectra into two groups. One group, containing methyl, cyclopropyl and adenosylcobalamin, has a maximum at 458-460nm. The other group, containing isopropyl, isobutyl, neopentyl, cyclobutyl, cyclopentyl and cyclohexylcobalamin, has a maximum at 438-442nm. The spectra of ethyl and n-propylcobalamin are intermediate between the two groups, showing bands of similar intensity at 440-441nm and 455nm. Thus changing the organoligand for the 5-coordinate alkylcobalamins produces maxima in only one of two regions (~440 and ~460nm) and has a marked effect on the ratio of the extinction coefficients

at 440nm and 460nm. This contrasts with the changes seen when ligands are varied in the 6-coordinate normal cobalamins where a gradual shift in the wavelength of the αβ bands is seen but there is little change in the overall shape of the bands.[5i]

The appearance of only two maxima, together with the variation in intensity of the maxima in the series suggests the possibility that two species are in equilibrium for each alkylcobalamin. In order to check this possibility spectra of ethylcobalamin in 0,1M sulphuric acid at pH = 1,0 were run at concentrations of $2,0 \times 10^{-4}$M, $2,0 \times 10^{-5}$M, $2,0 \times 10^{-6}$M. The ratio of the extinction coefficients of the bands at 440 and 455nm ($1,08 \pm 0,02$) did not vary over this range. Also, there was no change in the spectra of methyl, ethyl, cyclopropyl and cyclobutylcobalamin on heating to 80°C and for isopropyl, isobutyl, neopentyl, cyclopentyl and cyclohexylcobalamin on heating to 50°C. It therefore appears that only one species ($\geq$ ninety-five per cent) is present at pH = 1 for each alkylcobalamin (or that $\Delta H = 0$ for interconversion of the species.

Using the variation of the ratio $\varepsilon 440 : \varepsilon 460$ the alkylcobalamins can be placed in the following order for the effect of the different alkyl ligands on the cobalt and hence on the corrin ring:- methyl ~ adenosyl < cyclopropyl $\leq$ 3-butenyl < cyclopropylmethyl $\leq$ n-propyl $\leq$ ethyl < isobutyl < cyclobutyl < isopropyl ~ neopentyl ?cyclopentyl $\leq$ cyclohexyl. A similar order is obtained from equilibrium studies (see later).

There are a large number of overlapping bands in the 325-400nm region of the spectra of alkylcobalamins. Only the band at ~380nm (the band of highest wavelength) is considered. The wavelengths and extinction coefficients of this band are probably altered by the presence of other bands close by. This band gradually shifts to longer wavelengths as the ligand is changed in the order methyl ∽ cyclopropyl < adenosyl<3-butenyl<cyclobutyl< ethyl∽ n-propyl < cyclopropylmethyl < isopropyl ≃ cyclopentyl < cyclohexyl ≃ isobutyl < neopentyl. The behaviour of the band at approximately 380nm is different from that of the β band in that it shows a gradual shift to higher wavelengths rather than a jump to lower wavelengths. The higher wavelength of this band in isobutyl and neopentylcobalamin is the only parameter which distinguishes isobutyl from cyclobutylcobalamin and neopentyl from cyclopentylcobalamin.

The band at 303nm again behaves differently. The wavelength of this band does not change for the entire series of organocobalamins but for series 1 and 3 there is a gradual increase in intensity of this band.

The origin of the changes in the spectra is obscure but, however these changes originate, they are useful for establishing an order for the effects of different alkyl ligands on the central cobalt atom.

4.2.3 Spectra of organocobinamides

The wavelengths and ratios of extinction coefficients at

440nm and 460nm are given in Table 4.2 for organocobinamides (G) forms.

Table 4.2 - UV-visible Spectra of Organocobinamides

| Ligand | αβ region | | | γ region |
|---|---|---|---|---|
| | Maxima λ/nm | | Ratio A440:A460 | Maximum λ/nm |
| methyl | 460 | | 0,87 | 374 |
| ethyl[a] | 455 | 440 | 1,02 | 380 |
| isopropyl[b] | 460 | 442 | 1,27 | 382 |
| ethyl[a] | 455 | 440 | 1,02 | 380 |
| n-propyl | 455 | 441 | 1,01 | 381 |
| isobutyl | | 440 | 1,03 | 385 |
| neopentyl | | 438 | 1,06 | 388 |
| cyclobutyl | | 438 | 1,03 | 378 |
| cyclohexyl[c] | | 440 | 1,25 | 384 |

[a] ref 39

[b] ref 21

c ref 23

The spectrum of cyclobutylcobinamide is given in Fig.4.5. Except for cyclobutylcobinamide, isobutylcobinamide and neopentylcobinamide where the ratio ε440 :ε460 is lower than for the corresponding cobalamins the spectra of the cobinamides are identical to those of the cobalamins in acidic solution. For the cobinamides the ligand order (on the basis of ε440:ε460) is

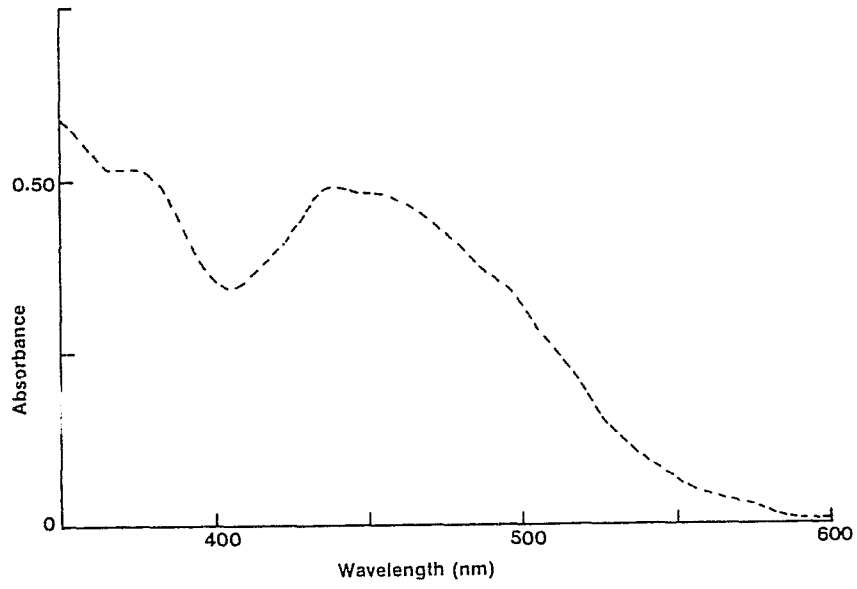



Fig.4.5 Spectrum of a 6,0 x $10^{-5}$M solution of the (G) form of cyclobutylcobinamide.

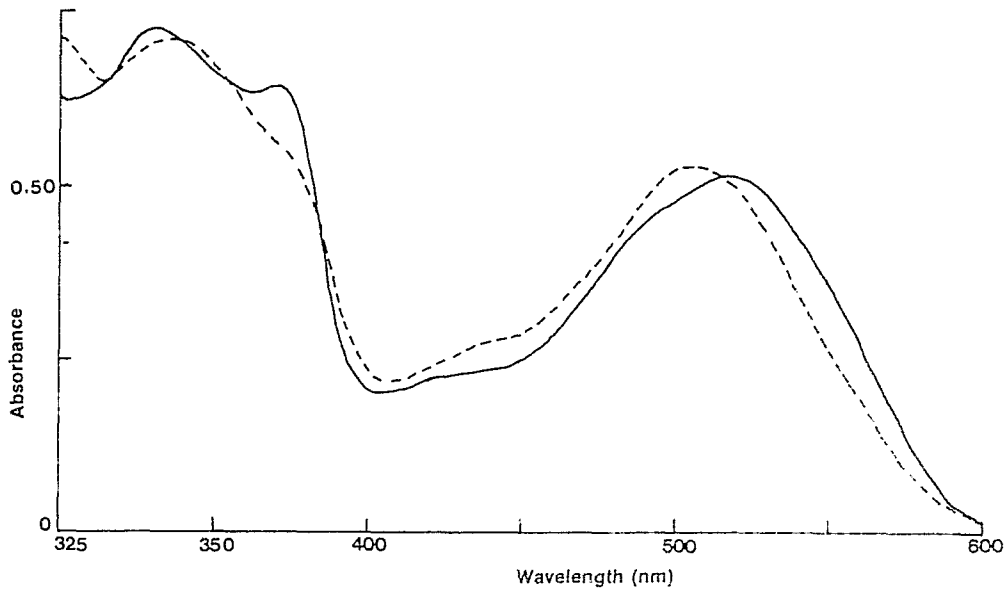



Fig.4.6 Spectra of 6,0 x $10^{-5}$M solutions of the 'base-on' (A) forms of methyl (—— ) and ethyl (---) cobalamin.

methyl < ethyl ~ n-propyl ~ isobutyl ~ cyclobutyl ~ neopentyl < isopropyl ~ cyclohexyl. The series is slightly different (for isobutyl to neopentyl) from the corresponding series for the protonated, base-off organocobalamins.

4.2.4 Spectra of 'base-on' organocobalamins

Because alkylcobalamins in neutral solution show an equilibrium between the 6-coordinate base-on (A) form and the 5-coordinate base-off form (C) it is impossible to obtain spectra of the pure 6-coordinate forms (section 5.3). For this reason only the spectra of alkylcobalamins which have ≥ eighty-five per cent of the 6-coordinate (A) form present at room temperature and neutral pH, viz. methylcobalamin, ethylcobalamin, n-propylcobalamin, cyclopropylcobalamin, cyclopropylmethylcobalamin, 3-butenylcobalamin, adenosylcobalamin will be considered. The UV-visible spectra for methylcobalamin and ethylcobalamin are given in Fig.4.6 and the wavelengths and extinction coefficients are tabulated in Table 4.3

The absorption bands in the αβ region are found at longer wavelengths for the red base-on forms than for the yellow protonated base-off forms. The bands are found at higher wavelengths (506-522nm) for the base-on forms than for the base-off forms (438-460nm) and the bands for the base-on forms have broader maxima. There is a gradual shift to shorter wavelength in the order cyclopropyl ~ adenosyl ≤ methyl < n-propyl ~ 3-butenyl ~ cyclopropylmethyl ≤ ethyl instead of the discontinuous shift seen in the protonated base-off forms.

Table 4.3 - UV-visible Spectra of 'base-on' (A) Forms of Organocobalamins

| Ligand | Maxima: $\lambda$ /nm($10^{-4}\varepsilon$) | | | |
|---|---|---|---|---|
| | $\alpha\beta$ region | $\gamma$ region | further UV region | |
| adenosyl | 522 (0,84) | 374 (1,1) | 340 (1,2) | 305 (1,3) |
| methyl | 518 (0,87) | 372 (1,1) | 342 (1,3) | 310 (1,3) |
| ethyl | 506 (0,91) | 375 (0,97) | 342 (1,3) | 305 (1,7) |
| n-propyl | 510 (0,95) | 375 (0,97) | 343 (1,3) | 305 (1,6) |
| cyclopropyl | 522 (0,87) | 375 (0,95) | 342 (1,4) | 305 (1,3) |
| 3-butenyl | 510 (0,84) | 375 (0,92) | 342 (1,2) | 305 (1,4) |
| cyclopropylmethyl | 510 (0,83) | 375 (0,98) | 342 (1,3) | 305 (1,5) |

In the $\gamma$-region methylcobalamin, cyclopropylcobalamin and adenosylcobalamin have well-defined peaks at 372-375nm. The other alkylcobalamins have this peak reduced to a shoulder on the peak at 342-343nm. The wavelengths and the extinction coefficients of the $\gamma$band may be affected by the large number of overlapping bands in the region below 400nm. In ethyl and n-propylcobalamin the real wavelength of the shoulder for this reason is probably closer to 380nm than the 375nm quoted in Table 4.3

In the region around 305nm the absorbance is significantly lower for methyl, cyclorpropyl and adenosylcobalamin than for ethyl and n-propylcobalamin (even when absorbance due to the base-off forms of ethyl and n-propylcobalamin is taken into account). This correlates with changes in wavelength in the $\alpha\beta$ region.

## 4.3 pH-Dependent equilibria of organocobalamins

Measurement of the pKa for the protonation and displacement from coordination of the benzimidazole side-chain of cobalamins is a useful method for studying the effect of changing the 'upper' axial ligand since the pKa for this equilibrium is very sensitive to the nature of the axial ligand.[5j ,58] The value of this pKa has been correlated with the σ donor power of the upper ligand for a large number of ligands where the donor atom is C, N, O, S, Cl, I. For these ligands the pKa increases with increasing σ donor power.[58] The same occurs for the series of cobalamins where the ligand is a carbanion (viz. cyanide, ethinyl, vinyl, methyl) where the pKa rises with increasing σ donor power of the ligands[58] and is not significantly affected by the steric effect of the ligand. The order of ligands observed is the same as that for the wavelength of the αβ and γ bands in the UV-visible spectra of the cobalamins and for the NMR resonance of the $C_{10}$ hydrogen in the corrin ring. Thus the effect on the axial ligand *trans* to the donor ligand (the *trans* effect) and the effect on the corrin ring (the *cis* effect) must have a common origin; both the *trans* and the *cis* ligands 'see' the electron density on the cobalt atom. These results show that the groups in the 'upper' position affect the pKa by their ability to withdraw or donate electron density to the central cobalt atom.[58]

On a more quantitative basis, it has been shown that there is a linear relationship between the pKa for the displacement of

benzimidazole when the upper ligand is a substituted ethyl group ($Co\text{-}CH_2CH_2R$) or substituted methyl group ($Co\text{-}CH_2R$) and the Hammet meta-substituent of R.[59]

For bulky alkyl ligands in the upper coordination position the protonated 'base-off' five-coordinate form should be stabilized relative to the six-coordinate 'base on' form. This should result in an increase in the pKa with increasing steric bulk of the upper axial ligand. In order to assess the effects of steric compression (caused by the upper axial ligand) on the pKa as opposed to the inductive effects of the upper ligand, it was decided to study the effect on the pKa by the ligands in series 1, 2 and 3 discussed in section 4.1.2.

4.3.1 Determination of the pKa's of organocobalamins

UV-visible spectra were run at pH's = 1, 6-7 and 12 at room temperature for all the organocobalamins prepared. In all cases the spectra at pH = 6-7 were identical to those at pH = 12 and the only spectroscopically observed equilibrium was that between the yellow, protonated 'base-off' form and the unprotonated 'base-on' forms at pH's between 2 and 5.

For organocobalamins where a tetrahedral carbon atom is bound to the cobalt atom the protonated base-off species with $H_2O$ coordinated to the cobalt (D) can be neglected since ≥ ninety-five per cent of the five-coordinate, protonated 'base-off' (E). form is present (section 4.2.2). By analogy the unprotonated base-off species with $H_2O$ coordinated to the cobalt (B) can be

neglected. Thus the pH-dependent equilibrium is between forms A and C in neutral solution and form E in acidic solution and the pKa can be given by

$$pKa = \frac{[H^+]([A] + [C])}{[E]}$$

The pKa values for cyclopropyl, cyclobutyl, isobutyl, cyclopropylmethyl and 3-butenylcobalamin were determined by spectrophotometric titration[43] at 25°C and are given in Table 4.4. In each case the number of protons involved in the equilibrium was found to be equal to one. For the unstable alkylcobalamins, isopropyl , neopentyl , cyclopentyl and cyclohexylcobalamin the pKa values are much less accurate and for isopropyl and cyclopentylcobalamin only a limiting value could be determined. These pKa values are also listed in Table 4.4

For the spectrophotometric titration of the stable alkylcobalamins, viz. cyclopropyl , cyclobutyl , isobutyl , cyclopropylmethyl and 3-butenylcobalamin, $3 - 5 \times 10^{-5}$M solutions in 0,05 M sulphuric acid contained in a 1,0cm spectrophotometer cell were used. These were titrated with solutions of 0,1 - 1,0M sodium hydroxide as described in section 2.2.6. The ionic strength of the solution was not kept constant. The ionic strength of the solution appeared to have no effect on the spectrum of organocobalamins. For methylcobalamin at pH = 7,4 increcsing the ionic strength from $\mu = 0$ (deionized water to $\mu = 0,5$ (0,5M sodium perchlorate) and at pH = 1,0 increasing the ionic strength from $\mu = 0,15$ 0,05M sulphuric acid to $\mu = 0,65$

Table 4.4 - Values of pKa for Organocobalamins

| Ligand | Present results[a] | | Other results[c] | | |
|---|---|---|---|---|---|
| | pKa[b] | no. of $H^+$ | pKa[b] | No. of $H^+$ | ref |
| adenosyl | | | 3,3 - 3,5 | 1 | 5b |
| methyl | | | 2,5 - 2,7 | 1 | 5b |
| ethyl | | | 3,87 | ? | 5b |
| isopropyl | $\geq$ 4,5 | ? | | | |
| ethyl | | | 3,87 | ? | 5b |
| n-propyl | | | 3,84 | ? | 5b |
| isobutyl | 4,15 $\pm$ 0,05 | 1 | 4,20 | ? | 24 |
| neopentyl | 4,7 $\pm$ 0,02 | ? | 4,55 | ? | 24 |
| cyclopropyl | 2,82 $\pm$ 0,06 | 1 | 3,71 | ? | 24 |
| cyclobutyl | 4,17 $\pm$ 0,04 | 1 | 3,83 | ? | 24 |
| cyclopentyl | $\geq$ 4,5 | ? | | | |
| cyclohexyl | 4,7 $\pm$ 0,02 | ? | | | |
| 3-butenyl | 3,42 $\pm$ 0,07 | 1 | | | |
| cyclopropyl-methyl | 3,88 $\pm$ 0,08 | 1 | | | |

[a] $Ka = \frac{[H^+]\ ([A]+[C])}{[E]}$

[b] Conditions - 25°C, ionic strength not held constant

[c] Conditions - various, see original papers

(0,05M sulphuric acid + 0,5M sodium perchlorate) caused no noticeable change in the spectra. The titrations were monitored by measuring the change in absorbance (at 440nm for cyclopropylcobalamin, 510nm for cyclobutylcobalamin, 439nm for isobutylcobalamin, 510nm for cyclopropylmethylcobalamin and 444nm for 3-butenylcobalamin) for each addition of sodium hydroxide and the pH was measured using a glass microelectrode. Good isosbestic points were obtained in each case.

The changes in optical density were evaluated in terms of two species using the equation

$$pKa = pH + \log \left( \frac{|A_1 - A|}{|A - A_2|} \right)$$

where $A_1$, $A_2$ and A represent the absorbance at a particular wavelength $\lambda$ at the start of the titration, the end of the titration and any pH during the titration, respectively. All absorbance values were corrected for the volume change on the addition of sodium hydroxide. Graphs of $\log \left( \frac{|A_1 - A|}{|A - A_2|} \right)$ vspH gave slopes of 1, indicating that only one proton is involved. The pKa's were calculated by solving the above equation for pH values close to the pKa value. The experimental plots for cyclopropyl , cyclobutyl , isobutyl , cyclopropylmethyl and 3-butenylcobalamin are given in Fig.4.7.

Accurate pKa values could not be determined for isopropyl , neopentyl , cyclopentyl and cyclohexylcobalamin for two reasons.

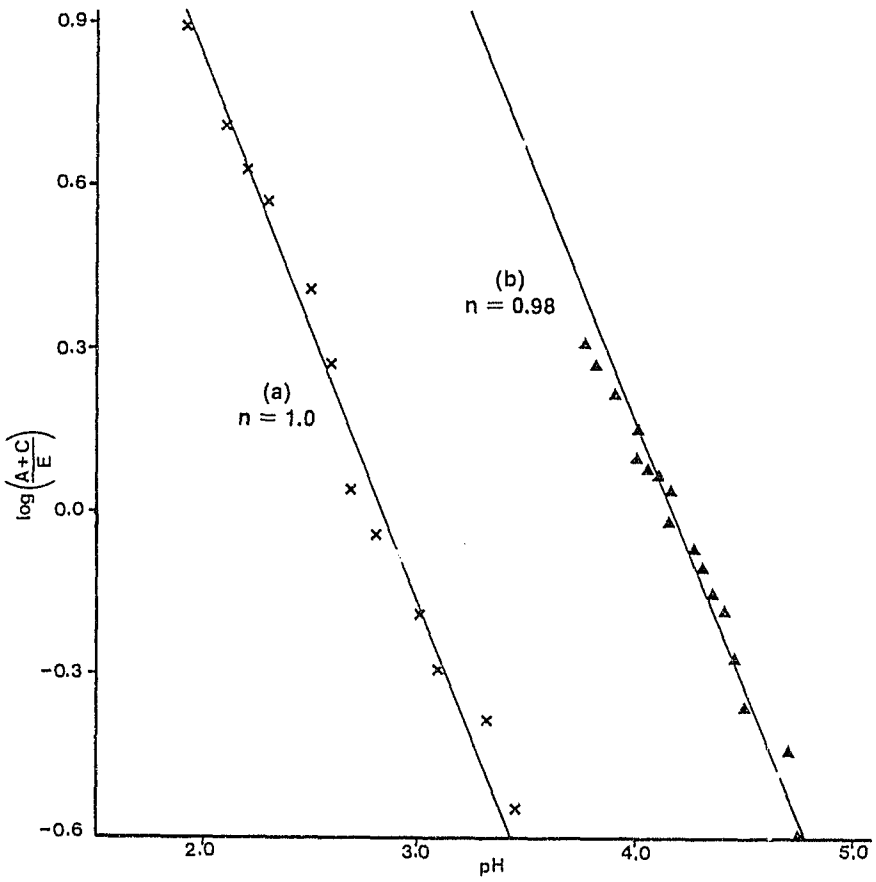
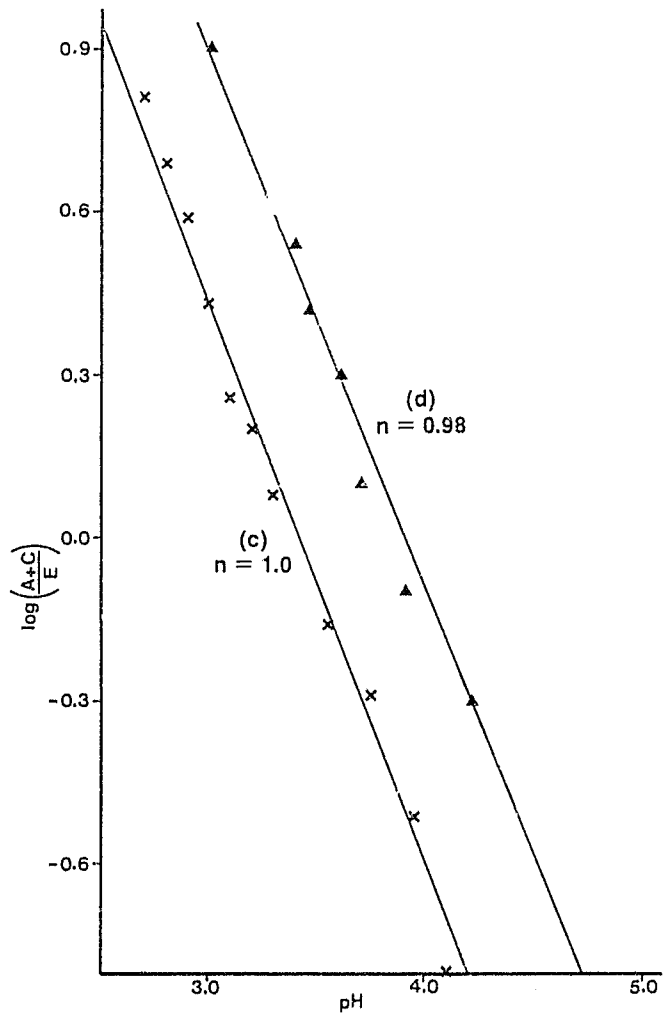
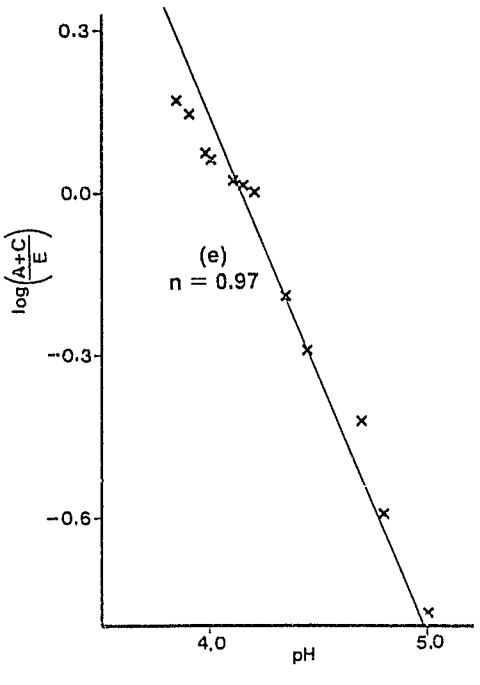



Fig.4.7 Experimental plots for determining the values of pKa and n (number of protons) for (a) cyclopropylcobalamin, (b) cyclobutylcobalamin, (c) 3-butenylcobalamin, (d) cyclopropylmethylcobalamin and (e) isobutylcobalamin.


0.9
0.6
0.3
0.0
-0.3
-0.6
log((A+C)/E)
(c)
n = 1.0
(d)
n = 0.98
3.0
4.0
5.0
pH


0.3
0.0
-0.3
-0.6
log((A+C)/E)
(e)
n = 0.97
4.0
5.0
pH

Firstly, the differences between the spectra at pH = 1 and at neutral pH are much smaller than for cyclopropylcobalamin etc., particularly for isopropylcobalamin and cyclohexylcobalamin. This is due to the presence of larger amounts of the 'base-off' unprotonated form C, which has a very similar spectrum to that of the 'base-off' protonated (E) form (section 5.3). Secondly, these alkylcobalamins are unstable, especially isopropylcobalamin and cyclopentylcobalamin, and their instability increases with increasing pH.

Because of their instability it was not possible to determine the pKa values for these alkylcobalamins by the above method. For isopropyl-, cyclopentyl- and cyclohexylcobalamin a series of solutions at the same concentration ($5,5 \times 10^{-5}$M) but at pH's varying from 2 - 9 were prepared as described in section 3.4, using acetic acid of varying concentrations and acetic acid/ sodium acetate buffers (section 2.1.3) instead of phosphate buffer. For neopentylcobalamin a series of solutions of the same concentration ($6,8 \times 10^{-5}$M) but at pH's varying between 1 and 10 was prepared by the addition of dilute sulphuric acid or sodium hydroxide to a stock solution of neopentylcobalamin in $5 \times 10^{-4}$M sulphuric acid.

The neopentylcobalamin and cyclohexycobalamin solutions were handled under nitrogen. Ionic strength was not kept constant for these solutions. For each alkylcobalamin spectra

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were run at each pH used and the spectra superimposed, giving approximate isosbestic points. Plotting absorbance at $\lambda$=442nm for cyclohexylcobalamin and at $\lambda$=438nm for neopentylcobalamin versus pH gave approximate pKa values for these cobalamins (Table 4.4). Because of the very rapid decomposition of isopropyl and cyclopentylcobalamin only minimum values for their pKa's could be obtained (Table 4.4). For none of these cobalamins were the data sufficiently accurate to determine the number of protons involved in the equilibria but this is assumed to be one.

4.3.2 Ordering of alkyl ligands according to their pKa's

The pKa values of alkylcobalamins determined in this study as well as those determined by other workers are given for series 1, 2 and 3 in Table 4.3, which shows that even for organocobalamins where the ligand is coordinated via a tetrahedral carbon atom and the carbanion is an alkyl group without heteroatoms the pKa increases by about two logarithmic units in going from methyl- to neopentylcobalamin. This means that the equilibrium constant for the coordination of unprotonated benzimidazole decreases one hundred-fold. The data of Table 4.4 show that the pKa rises in the order: methyl (2,6) < cyclopropyl (2,8) < adenosyl (3,4) ~ 3-butenyl (3,4) < n-propyl (3,8) < ethyl (3,9) ~ cyclopropylmethyl (3,9) < isobutyl ~ cyclobutyl (4.2) < isopropyl, < neopentyl, cyclopentyl, cyclohexyl (all $\geq$ 4,5). In Table 4.5 the ligand orders for the various spectrophotometric parameters and the pKa's are summarized.

Table 4.5 Experimentally Observed Orders of Organoligands in Series 1, 2 and 3

| A. Observable parameter | Observed order of ligands |
|---|---|
| pKa | methyl≤cyclopropyl<adenosyl < ethyl~n-propyl <cyclobutyl ~ isobutyl<isopropyl~neopentyl~cyclopentyl~cyclohexyl |
| Spectra of E complexes in αβ region | methyl≤adenosyl<cyclopropyl < n-propyl≤ethyl < isobutyl<cyclobutyl<isopropyl~neopentyl (?cyclopentyl)≤cyclohexyl |
| Spectra of E complexes in γ region | methyl~cyclopropyl<adenosyl<cyclobutyl < ethyl~n-propyl ≤ isopropyl~cyclopentyl<cyclohexyl~isobutyl<neopentyl |
| Spectra of A complexes in α region | cyclopropyl~adenosyl≤methyl < n-propyl≤ethyl |
| Spectra of G complexes in αβ region | methyl<ethyl~n-propyl~isobutyl~cyclobutyl~neopentyl<isopropyl~cyclohexyl |

| B. Variable parameter of ligand structure | Observed order of ligands | | | |
|---|---|---|---|---|
| Substitution on Cα | methyl < | ethyl | | isopropyl |
| Substitution on Cβ | | ethyl~n-propyl < | isobutyl < | neopentyl |
| Size of alicyclic ring | cyclopropyl | | < cyclobutyl < | cyclopentyl≤cyclohexyl |
| cf also | adenosyl | | | |

4.3.3 pKa's of organocobalamins and inductive effects of organoligands

Two parameters useful as a measure of the inductive effect of a substituent are the Hammet meta constant ($\sigma m$) which is based on the equilibrium constants for the ionization of m substituted benzoic acids and the Taft constant ($\sigma^*$) which is based on the ratio of the rate of hydrolysis of acidic and basic esters.[60a,61] The pKa values for substituted acetic acids $RCH_2COOH$ correlate well with $\sigma^*$ and can therefore be used as a measure of the inductive effect of R.[60a,61]

For a series of organocobalamins where a minimum of steric interaction is expected, ie. the series where the ligand is cyanide, ethinyl, vinyl and methyl, there is a quantitative correlation between the effect of R on the pKa's for protonation of the benzimidazole base and the pKa's for the series of R substituted acetic acids.

| R = | N≡C- | HC≡C- | $CH_2$=CH- | $CH_3$ |
|---|---|---|---|---|
| pKa of $RCH_2COOH$[62,63] | 2,47 | 3,32 | 4,35 | 4,87 |
| pKa of cobalamin[51] | 0,1 | 0,7 | 2,4 (1,9) | 2,6 |
| difference | 2,4 | 2,6 | 2,0 (2,5) | 2,3 |

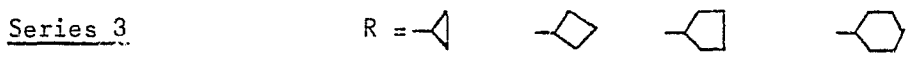
The correlation is surprisingly good considering that the nature of the equilibria varies over the series of organocobalamins. For cyanocobalamin and probably ethinylcobalamin the pKa represents a simple equilibrium between the 6-coordinate 'base-on' form (A) and the protonated 'base-off' (D) form.[5k,21] For

vinylcobalamin seventy per cent of the 5-coordinate (E) form is present in acid[21] and correction for this gives the pKa value (in parentheses, above) for the equilibrium between the 6-coordinate A and D forms.

The correlation breaks down completely beyond methylcobalamin as is shown for series 1,2 and 3 (where steric interaction is expected).

| Series 1 | R = $CH_3$- | $CH_3CH_2$- | $(CH_3)_2CH$- |
|---|---|---|---|
| pKa of $RCH_2COOH$[62] | 4,87 | 4,82 | 4,78 |
| pKa of cobalamin[51] | 2,6 | 3,9 | $\geq$ 4,5 |
| difference | 2,3 | 0,9 | $\leq$ 0,3 |

| Series 2 | R = $CH_3CH_2$- | $CH_3CH_2CH_2$- | $(CH_3)_2CHCH_2$- | $(CH_3)_3CCH_2$- |
|---|---|---|---|---|
| pKa of $RCH_2COOH$[62] | 4,82 | 4,86 | 4,78 | ? |
| pKa of cobalamin[51] | 3,9 | 3,9 | 4,2 | 4,7 |
| difference | 0,9 | 1,1 | 0,6 | ? |

| Series 3 | R =  | | | |
|---|---|---|---|---|
| pKa of $RCH_2COOH$[64,65] | 4,74 | ? | 4,84 | 4,87 |
| cobalamin[51] | 2,8 | 4,2 | $\geq$ 4,5 | 4,7 |
| difference | 1,9 | ? | $\leq$ 0,3 | 0,2 |

Substitution of the methyl group by other saturated alkyl and cycloalkyl groups results in a change of $\geq$ 2 in the pKa of the organocobalamins but of only $\leq$ 0,1 in the substituted acetic acids.

The same breakdown in correlation between the pKa's of organocobalamins where steric hindrance occurs is observed for

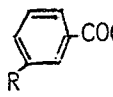
other inductive parameters eg. the pKa's of substituted formic acids (R-COOH),[62,66] which correlate with σ*; the pKa's of meta substituted benzoic acids,[67,68] which correlate with σm and the pKa values for the removal of a proton from alkanes (RH)[69] which give a direct measure of the stability of the carbanion $R^-$ (Table 4.6).

It seems reasonable to conclude that the breakdown in correlation between the pKa's of organocobalamins beyond methylcobalamin and the various inductive parameters is caused by the increasing importance of steric relative to indictive effects for these cobalamins. Any inductive effects that may occur are swamped by the much longer steric effects.

## 4.4 Equilibria of organocobinamides with imidazole

When the methyl ligand in methylcobalamin is substituted by n-propyl[70] or cyclohexyl[22,23] not only is the pKa increased, ie. the binding constant for the benzimidazole base decreased, but the binding constants for other ligands with the corresponding cobinamides are decreased. The equilibrium constants for the binding of imidazole, ammonia and cyanide by methylcobinamide are 11, 0,1 and $230M^{-1}$ respectively but the equilibrium constant for the binding of imidazole by n-propylcobinamide is as low as $0,1M^{-1}$ [70]. The spectrum of cyclohexylcobinamide remains unchanged in the presence of imidazole,[22] ammonia, cyanide and other ligands (methylamine, ethylamine and pyridine)[23] indicating that the binding constants for cyclohexylcobinamide with these ligands must be very low.

Table 4.6 - pKa's of Organocobalamins Compared to Various Inductive Parameters

| Ligand | pKa of R-COOH[a] | pKa of  [e] | pKa of R-H[h] | pKa of organocobalamin[i] |
|---|---|---|---|---|
| N ≡ C- | -[b] | 4,27[f] | 9,1 | 0,1 |
| HC ≡ C- | 1,84[c] | | 25 | 0,7 |
| $H_2C = CH_2$- | 4,25[c] | | 36,5 | 2,4 (1,9) |
| $CH_3$ | 4,76[c] | 5,60[f] | 40 | 2,5 - 2,7 |
| $CH_3$- | 4,76[c] | 5,60[f] | 40 | 2,5 - 2,7 |
| $CH_3CH_2$- | 4,87[c] | 5,64[f] | 42 | 3,9 |
| $(CH_3)_2CH$- | 4,86[c] | 5,71[f] | 44. | ≥ 4,5 |
| $CH_3CH_2$- | 4,82[c] | 5,64[f] | 42 | 3,9 |
| $CH_3CH_2CH_2$- | 4,82[c] | | 44 | 3,8 |
| $(CH_3)_2CHCH_2$- | 4,84[c] | | | 4,2 |
| $(CH_3)_3CCH_2$- | 4,79[c] | 5,67[f] | 44 | 4,7 |
|  | 4,82[d] | 5,85[g] | 39 | 2,8 |
|  | 4,79[d] | 5,89[g] | 43 | 4,2 |
|  | 4,99[d] | 5,93[g] | 44 | ≥ 4,5 |
|  | 4,90[d] | 5,93[g] | 45 | 4,7 |

a. in water

b. N ≡ C-COOH is unstable

c. ref 62

d. ref 66

e. in water/ethanol 50/50

f. ref 67

g. ref 68

h. ref 69

i. From table 4.3 and ref 51.

4.4.1 Binding constant for neopentylcobinamide with imidazole

It was decided to determine the binding constant of neopentylcobinamide with imidazole because no quantitative value exists for the extent of binding of imidazole by an organocobinamide corresponding to an organocobalamin with a high pKa.

A limiting value for the binding constant of neopentylcobinamide with imidazole was determined by comparing the spectra of neopentylcobinamide in the presence and absence of 3,3M imidazole at pH = 9. It was assumed that the imidazole complex should have a similar spectrum in the 400-550nm region to that of a typical 6-coordinate organocobalamin. There was no significant difference between the neopentylcobinamide spectra with and without imidazole which means that less than ten per cent of the neopentylcobinamide is coordinated to imidazole under these conditions. The value of the equilibrium constant can therefore be given as

$$K = \frac{[NP\text{-}Co\text{-}imid]}{[NP\text{-}Co]\,[imid]} < 3 \times 10^{-2} M^{-1}$$ and the binding constant as

$$\log K < -1,5$$

NP = neopentyl
imid = imidazole

Neopentylcobinamide, in the presence of imidazole, is slowly decomposed (section 6.4.5), via the intermediate formation of the imidazole adduct but this decomposition is too slow to obscure any initial changes in spectrum.

The binding constants (log K) for the coordination of imidazole by organocobinamides therefore decrease in the order: methyl (+ 1,0) > n-propyl (- 1,0) > neopentyl (< - 1,5). The binding constant for cyclohexylcobinamide with imidazole is

probably of the same order of magnitude as that for neopentylcobinamide.

## 4.5 Discussion

As shown in Table 4.5, for each series of alkylcobalamins there is a parallel between the orders determined for the A and E forms from their UV-visible spectra and that determined from their pKa's and this order is determined by steric rather than by inductive effects (section 4.3.3). Series 1, 2 and 3 can be combined into a single series without any significant anomalies in the orders of spectra or pKa's and adenosylcobalamin can be given a definite position in this series. There is no significant difference between the effect of the two types of distortion of the Co-$\hat{C}_\alpha$-$C_\beta$ bond angle (increasing or decreasing Co-$\hat{C}_\alpha$-$C_\beta$) on the spectra and pKa's of organocobalamins and between the effects of primary and secondary organoligands.

### 4.5.1 Relief of steric strain in the 5-coordinate base-off organocobalamins

As shown in section 4.3.3 the pKa's of organocobalamins in series 1, 2 and 3 depend on steric, rather than inductive, effects. The pKa's in the series increase in the order methyl < ethyl < isopropyl, ~ethyl n-propyl < isobutyl < neopentyl, cyclopropyl < cyclobutyl < cyclopentyl ≤ cyclohexyl. This shows that the five-coordinate, prononated, 'base-off' form (E) becomes increasingly stable relative to the six-coordinate, unprotonated 'base-on' (A) form as steric compression by the organdigand is increased.

Removal of the benzimidazole base from coordination could stabilize the organocobalamin by two possible mechanisms, viz.

movement of the cobalt atom out of the plane of the ring[23] or the 'bending down' of the corrin ring,[24] both of which should result in a decrease in steric compression. The suggestion that large alkyl substituents attached to cobalt should 'bend' the corrin ring downwards was made by Grate and Schrauzer.[24] They considered the fact that in cobalamin derivatives there is contact between the hydrogen atom on carbon B-4 of benzimidazole and the C5 and C6 carbons of the corrin ring, causing the corrin ring to bend towards the upper axial ligand, and suggested that when the upper axial ligand is bulky the corrin ring is 'bent downwards' causing the benzimidazole base to be removed from coordination. This explanation is not adequate because it cannot account for the effect of increasing steric compression by the organoligand on the spectra of the five-coordinate, prononated, 'base-off' (E) forms of organocobalamins. Also, ligands such as ammonia, cyanide and imidazole show a decrease in their equilibrium constants for coordination to organocobinamides with increasing steric bulk of the organoligand (section 4.4) which parallels the decrease in the equilibrium constant for coordination of unprotonated benzimidazole in the corresponding organocobalamins. These ligands should not cause bending of the corrin ring because of their much smaller steric bulk so the mechanism of Grate and Schrauzer cannot account for the observed effect of increasing steric bulk of the upper axial ligand on the equilibrium constants for coordination to organocobinamides.

Another possible mechanism for relief of steric strain in the five-coordinate 'base-off' organocobalamins, suggested by Brodie,[23] is movement of the cobalt atom out of the plane of the ring and the consequent increase in the $C_a$-$\overset{\wedge}{Co}$-Neq bond angle. If alkyl derivations of corrinoids and model compounds for corrinoids are considered it can be seen, using X-ray diffraction, that in the six-coordinate, octahedral complexes the cobalt ion lies within the plane formed by the four equatorial ligand atoms,[52] but that in the five-coordinate, square pyramidal complex $CH_3Co(BAE)$ (BAE = bis (acetylacetone)(ethylenediamine) the cobalt is displaced 0,12Å above the plane of the equatorial ligand towards the carbon atom of the single axial ligand.[71] This allows a shortening of the Co-C bond length from 1,99 - 2,05Å in the six-coordinate complexes[52] to 1,95Å in the 5-coordinate complex[71] and presumably serves to reduce steric repulsion. For the iodo - Co(II) cobyrinic acid heptamethyl ester dimer,[72,73] where two symmetrical halves consisting of a cobalt atom surrounded by a corrin ring are joined by an iodine atom, each cobalt atom is displaced from the plane of the corrin ring by an average of 0,12Å (0,11 and 0,13Å). Thus the displacement of cobalt from the plane of the ring is possible for both cobalt corrinoids and their model compounds. Unfortunately, there is no X-ray crystallographic data on any of the presumed five-coordinate organocorrinoids so it is not possible to say for certain that the Co atom is displaced above the plane of the ring.

However, the suggestion that the cobalt atom is positioned above the plane of the ring in the five-coordinate, 'base-off' corrinoids but is in the plane of the ring for the six-coordinate 'base-on' corrinoids provides a simple and reasonable explanation for the decrease in steric compression and the increase in stability for the five-coordinate organocobalamins as compared to the six-coordinate organocobalamins.

### 4.5.2 Relief of steric strain in the six-coordinate 'base-on' organocobalamins

For the six-coordinate 'base-on' organocobalamins the mechanism for relief of steric strain in the five-coordinate organocobalamins (movement of the cobalt atom out of the plane of the ring) proposed in section 4.5.1 is not available.

X-ray crystallography of adenosylcobalamin also shows that the $C_\alpha$- $\hat{Co}$-Neq bond angles (89°, 93°) are not distorted although the Co-$\hat{C}_\alpha$-$C_\beta$ bond angle is 125° (rather than 109,5°) and the Neq-$\hat{Co}$-NBzm bond angles (88° and 92°) are not distorted although the benzimidazole (coordinated to cobalt) is tilted with Co-$\hat{N}$Bzm-C bond angles of 123° and 132° (due to contact between the hydrogen atom on carbon $B_4$ of benzimidazole and the C5 - C6 portion of the corrin ring).[47] This means, if it is assumed that the structure of other organocobalamins is similar to adenosylcobalamin, that steric compression around the donor atom of the upper axial ligand cannot be alleviated by changing the $C_\alpha$-$\hat{Co}$-Neq bond angles in 6-coordinate 'base-on' organocobalamins.

This makes sense if the high electron density around the Co-$C_\alpha$ bond from the lone pairs of electrons on the cobalt and the equatorial nitrogen atoms is taken into consideration.

There must be some other mechanism of relief of steric strain operative in the 6-coordinate organocobalamins. As shown in the following section the other possible mechanism for relief of steric strain is the lengthening of the Co-$C_\alpha$ bond.

4.5.3 Secondary inductive effect in sterically hindered organocobalamins-proposal of a long Co-C bond

The order of ligands for the spectra of organocobalamins (both in the 'base-on' and 'base-off' forms) is very similar to that for the pKa's for all the ligands considered. These results show that changes in the structure of ligands are 'perceived' on the whole as changes in a single variable by both the cis (corrin) ligand and the trans (benzimidazole) ligand and hence also by the cobalt atom. Both the corrin ligand and the benzimidazole base 'see' the electron density on the cobalt atom and the variable 'seen' by the cis and trans ligands, although steric in origin, must be ultimately electronic. This variable is almost certainly the Co-C bond length. In organocobalamins where there is a large degree of steric interaction between the alkyl ligand and the corrin ring the lengthening of the Co-C bond will provide relief of steric strain. The lengthening of the Co-C bond with increasing steric compression by the organoligand can only be proved conclusively by X-ray crystallography of a suitable series of alkylcobalamins.

For organocobaloximes (cobalamin model complexes) X-ray crystallography shows a positive correlation between the steric effect of the organoligand and the length of the Co-C bond. Marzilli et al[74] determined the structure of trans - bis (dimethylglyoximato) - (isopropyl)(pyridine) cobalt (III) and showed that it had an unusually long Co-C bond. They showed that there was a definite linear relationship between the Co-C bond length and the number of substituents on the methyl group for the series of cobaloximes py $Co(DH)_2R$ where R = $-CH_3$,[75] $-CH_2COOCH_3$[76] and $-CH(CH_3)_2$. The idea of the long Co-C bond in adenosylcobalamin and in other organocobalamins where steric compression occurs around the Co-C bond is thus well supported by model studies.

The increase in length of the Co-C bond must be accompanied by a change in the electron distribution in the partially covalent Co-C bond and by a change in the hybridization of the 3d, 4s and 4p orbitals of the cobalt.

The ratio of the absorbance for the $\alpha\beta$ band at 440nm and 460nm ($\varepsilon 440/\varepsilon 460$) puts all the ligands studied in an order according to the steric compression they cause. The change in the ratio $\varepsilon 440/\varepsilon 460$ is probably associated with increasing movement of the cobalt out of the plane of the ring but since the lengthening of the Co-C bond must be intimately associated with the movement of the Co out of the plane of the ring the ratio $\varepsilon 440/\varepsilon 460$ probably also is a reflection of the Co-C bond length.

4.5.4 Comparison of effects of substitution at $C_\alpha$ and $C_\beta$

Although the effects of substitution at $C_\alpha$ and $C_\beta$ are fundamentally the same there are minor differences in the spectra of alkylcobalamins with substitution at $C_\alpha$ and $C_\beta$.

The band appearing at ~380nm in the spectra of 5-coordinate (F) organocobalamins shows an order less similar to the pKa order over the entire group of alkylcobalamins studied. This band appears to be more sensitive to the substituents on $C_\beta$ than the $\alpha\beta$ band and is apparently the only variable which distinguishes neopentylcobalamin from isopropylcobalamin and cyclohexylcobalamin, and isobutylcobalamin from cyclobutylcobalamin.

The effect of substitution at the $\beta$ position on the spectra and pKa values appears to be smaller than the effect of $\alpha$-substitution. If the pair of organocobalamins cyclopropylcobalamin and isopropylcobalamin (where the change of group from cyclopropyl to isopropyl occurs on the $\alpha$-carbon) is compared with cyclopropylmethylcobalamin and isobutylcobalamin (where the change of group from cyclopropyl to isopropyl occurs on the $\beta$-carbon) it can be seen that the magnitude of the effect both on the spectra and on the pKa ($\Delta \geq 1,7$ for $\alpha$-substitution and $\Delta = 0,45$ for $\beta$-substitution) is greater at the $\alpha$-position than at the $\beta$-position. The first two members of series 2 (where $\beta$-substitution occurs), ethylcobalamin and n-propylcobalamin show only very small differences in their spectra and pKa's.

## 4.6 Summary

The spectra in neutral and acid solution and the pKa's for protonation and displacement of the benzimidazole base were determined for organocobalamins with alkyl and cyclalkyl ligands having no heteroatoms. Increasing the amount of distortion around $C_\alpha$ (in the series methyl < ethyl < isopropyl and cyclopropyl < cyclobutyl < cyclopentyl < cyclohexyl) or $C_\beta$ (ethyl ≃ n-propyl < isobutyl < neopentyl) gives rise to parallel changes in the spectra and in the pKa's with no fundamental difference between primary and secondary groups, between alkyl and cyclalkyl groups (methyl ≡ cyclopropyl, ethyl ≡ cyclobutyl, isopropyl ≡ cyclopentyl and cyclohexyl) or between substitution on $C_\alpha$ and $C_\beta$ (neopentyl ≡ isopropyl). It is shown that the changes in spectra and pKa originate from steric rather than electronic effects of the organoligand. It is proposed that the main variable, as seen by the cobalt atom and the rest of the cobalamin molecule, is the Co-C bond-length and that relief of steric strain occurs by two mechanisms viz lengthening of the Co-C bond (in five- and six-coordinate complexes) and movement of the cobalt atom above the plane of the corrin ring (five-coordinate complexes).

## CHAPTER 5 - TYPES OF EQUILIBRIA SHOWN BY ORGANOCOBALAMINS

### 5.1 Introduction

The equilibria displayed by organocorrinoids are given in Fig.4.2. For organocobalmins there are three possible species in neutral aqueous solution. These are the six-coordinate 'base-on' form (A), the six-coordinate 'base-off' form with $H_2O$ coordinated in the sixth position (B) and the five-coordinate 'base-off' form (C). In acid solution there are two possible forms; the five-coordinate, protonated 'base-off' form (E) and the six-coordinate protonated 'base-off' form with $H_2O$ in the sixth coordination position (D). For vinylcobalamin all of these forms are found in solution.[21] However, for organocobalamins where the ligand atom bound to cobalt is a tetrahedral carbon atom only one species, the E form, is present ($\geq$ ninety-five per cent) in acid solution and there is no B form in neutral solution (section 4.2.2). Most organocobinamides considered here are entirely ($\geq$ ninety-five per cent) in the five-coordinate (G) form at room temperature but methylcobinamide has a small proportion ($\sim$ten per cent) of the six-coordinate form with $H_2O$ coordinated to the cobalt (F).[21]

No structure of a monomeric five-coordinate organocorrinoid has yet been determined using X-ray crystallography but $[CH_3Co\ BAE]$ has been shown by X-ray crystallography to be five-coordinate.[71] Evidence for five-coordination amongst organocobalamins, which

is based on that for the organocobinamides is adequately discussed elsewhere.[5k, 21]

As well as differences in the number and nature of the axial ligands organocorrinoids may also show differences in the conformation of the corrin ring, in the position of the cobalt atom with respect to the plane of the corrin ring and in subtle interactions between the corrin ring and the benzimidazole side-chain. Because of the large variety of different possible forms of organocobalamins and the complicated interactions within the organocorrinoid molecule the interpretation of the equilibria of organocorrinoids is difficult. In particular, difficulties arise in distinguishing between changes in conformation of the corrin ring and changes in coordination number. In this chapter the overall pattern of equilibria (detectable by UV-visible spectroscopy) of organocobalamins will be discussed. In section 5.2 it will be shown that changes in the conformation of the corrin ring and in the coordination number of organocorrinoids occur independently of each other and that changes in the conformation of the corrin ring have very little effect on the electronic spectra of organocorrinoids. The electronic spectra will therefore be used to elucidate the equilibria (involving changes in the number and nature of the axial ligands) of organocobalamins.

## 5.2 Conformation of the corrin ring

X-ray crystal structures have been determined for several

cobalt-containing corrinoids,[47,72,73,77-82] all of which can be placed in two groups according to the conformation of their corrin ring.[47] All the cobalamins so far studied ie. the two crystal forms ('wet'[77] and 'dry'[78]) of cyanocobalamin, cyano (13 epi) *cobalamin*[79] (neovitamin $B_{12}$), cyanocobalamin 5'-phosphate[80] and adenosylcobalamin[47] have one particular conformation of the corrin ring (which we have termed 'cobalamin'). For all the other corrinoids studied ie. cyanoaquocobyrinic acid,[81] 8-aminocobyrinic acid-C-lactam chloride cyanide,[82] and both halves of the iodide-bridged dimeric Co(II) complex of cobyrinic heptamethyl ester where the cobalt atom is five-coordinate and displaced by an average of 0,11 and 0,13Å from the mean plane the corrin ring[72,73] (all of which do not contain the benzimidazole base) the corrin ring is more nearly planar. This conformation we have termed 'normal'. The main difference between the 'normal' and 'cobalamin' forms of the corrin ring is that the 'cobalamin' forms show an obvious bend in the C5 - C6 region due to contact with the hydrogen atom on B-4 of the benzimidazole base.[47] Very similar conformations of the corrin ring are found when comparing pairs of cobalamins with differences in the conformations of the side-chains ('wet'[77] and 'dry'[78] cyanocobalamin), in the exocyclic ring (cyano(13 epi) cobalamin[79] and cyanocobalamin)[77,78] in the axial ligand and Co-N bond lengths (cyanocobalamin[77,78] and adenosylcobalamin[47]). The conformation of the corrin ring is therefore not greatly affected by changes in the side-chains, in

the exocyclic ring or in the axial ligands and can be considered independently of these changes.

The UV-visible thin film absorption and reflection spectra of cyano and adenosylcobalamin are similar to their absorption spectra in solution ie. these cobalamins have similar structures in the solid state and in solution.[21,42] The large differences between the UV-visible spectra of cyanocobalamin and adenosylcobalamin therefore show that large changes in electronic structure can occur without any significant change in the conformation of the corrin ring. Changes in the exocyclic ring (cf cyanocobalamin and cyano (13 epi) cobalamin[83] and in the side-chains[5i] do not have large effects on the UV-visible spectra.

5.2.1 Comparison of the spectra of compounds with the 'normal' and 'cobalamin' conformations

In order to establish whether or not changes in conformation alone can cause significant changes in the electronic structure (and hence in the UV-visible spectra) of corrinoids it is necessary to examine the spectra of pairs of corrinoids which (as far as possible) have the same axial ligands but different conformations of the corrin ring. The pairs chosen were methylcobalamin ('cobalamin' conformation) and (imidazole) methyl cobinamide ('normal' conformation) and cyanocobalamin ('cobalamin' conformation) and cyanoimidazolecobinamide ('normal' conformation). The imidazole adducts of both methylcobinamide[70] and cyanocobinamide[84] have been observed previously. The formation constants for the substitution of methylcobinamide and

aquocyanocobinamide respectively by imidazole are $11M^{-1}$ and $1,2 \times 10^{4}M$.[70,84]

The imidazole ligand was chosen for study because it is reasonably close in structure to, but not as bulky as, the benzimidazole ligand of cobalamins. Comparison of the two types of complex can however only give an approximate idea of the effect of change of conformation because the complexes with benzimidazole and imidazole probably differ in the degree of tilt of the coordinated base and in their Co-N bond lengths. Also, the pKa's of the two bases are different (pKa = 7,2 for imidazole and 5,0 for benzimidazole)[85,86] and imidazole is hydrophilic whereas benzimidazole is hydrophobic.

5.2.2 Results

Spectra of methylcobinamide in 0 - 10M imidazole in aqueous solution were run over the wavelength range 350-650nm. Comparison of the spectra of methylcobinamide in 10M imidazole solution and methylcobalamin in water (Fig.5.1) showed that the αβ bands of both nad maxima at 517-518nm. The shapes of the αβ bands are fairly similar but the spectrum of methylcobinamide in 10M imidazole has a more pronounced shoulder at 480nm than that of methylcobalamin. The γ bands are found at 373-374nm in both the cobinamide and the cobalamin. The spectrum of methylcobinamide in 10M imidazole consists of approximately ninety-nine per cent of the imidazole adduct (calculated from the formation constant) while the methylcobalamin spectrum consists of approximately

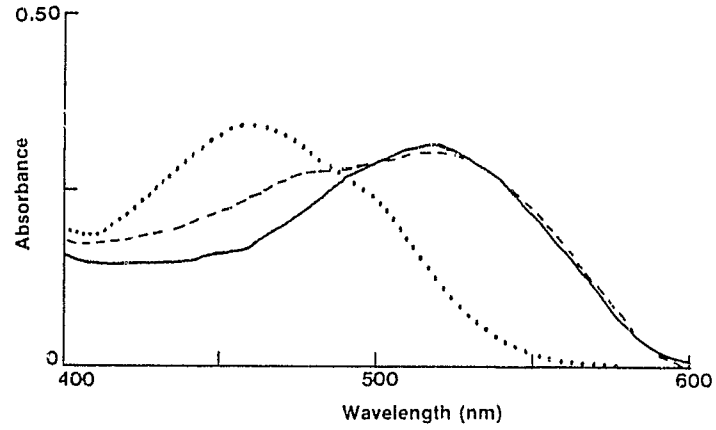



Fig.5.1 Spectra of methylcobinamide in water (...) and in 10M imidazole (---) and of methylcobalamin (—— ).

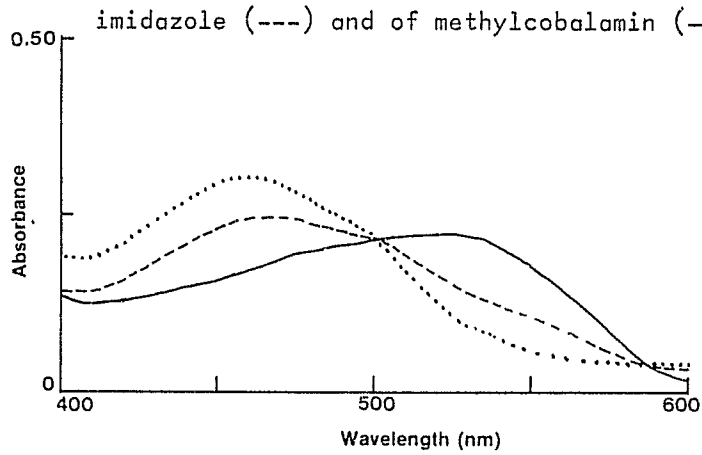



Fig.5.2 Spectra of methylcobinamide in 1M imidazole at pH = 8,24 (—— ), pH = 6,77 (---) and pH = 0,99 (...)

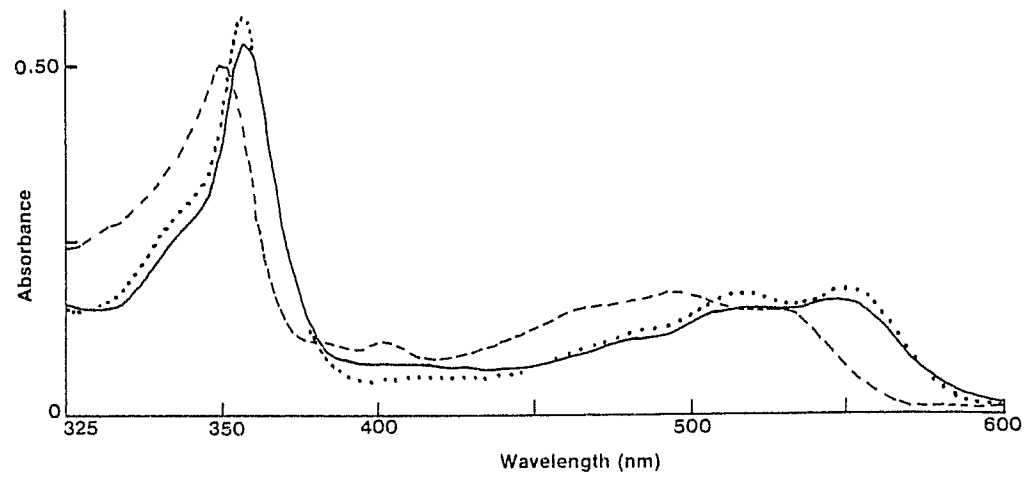



Fig.5.3 Spectra of aquocyanocobinamide in water (---) and in 1M imidazole (....) and of $B_{12}$ (—— ).

ninety-five per cent of the 'base-on' form and five per cent of the 'base-off' form (Table 5.1) which could possibly cause a slight displacement of the true maximum of the 'base-on' form to lower wavelengths. For methylcobinamide in 1M imidazole the concentration of the free base (pKa = 7,2[85]) was progressively decreased by the addition of concentrated sulphuric acid to lower the pH (Fig.5.2). An isosbestic point was observed at 500nm (cf. the acid/base titration of methylcobalamin where the corresponding isosbestic point occurs at 490nm. The 'solvent' effect of imidazole (due to alteration of the structure of water and changes in the hydrogen-bonding of the corrinoid side-chains) was small. The spectrum of methylcobalamin in 10M imidazole ($\gamma$ = 518nm for the $\alpha\beta$ band ) was very similar to that of methylcobalamin in water.

Spectra of aquocyanocobinamide in water, aquocyanocobinamide in 1M imidazole and cyanocobalamin in water (all at $1,8 \times 10^{-4}$M cobalt concentration were run from 325-650nm (Fig.5.3). For aquocyanocobinamide in imidazole, which consists of $\sim$99,99 per cent of the imidazole adduct, and cyanocobalamin the spectra are very similar. The $\alpha$, $\beta$ and $\gamma$ bands occur at 552nm, 520nm and 360nm for imidazole adduct of cyanocobinamide and at 550nm, 518nm and 361nm for cyanocobalamin (cf. aquocyanocobinamide-$\alpha$,$\beta$ and $\gamma$ bands at 525nm, 497nm and 352nm). Aquocyanocobinamide consists of two isomers[5m] but since their spectra are very similar this should make very little difference to any conclusions

Table 5.1 - Data for the Temperature-dependent Equilibria of Organocobalamins in Neutral Solution

| Ligand | Conversion of low-to high-temperature forms | | | | $x^a$ |
|---|---|---|---|---|---|
| | ΔH | | ΔS | | |
| | Kcal $mol^{-1}$ | KJ $mol^{-1}$ | cal $mol^{-1}K^{-1}$ | J $mol^{-1}K^{-1}$ | |
| adenosyl | | | | | ,10[b] |
| methyl | 3,6 ± 1 | 16 ± 5 | 7,5 ± 2 | 33 ± 10 | ,05 |
| ethyl | 3,8 ± 0,5 | 16 ± 2 | 10 ± 2 | 43 ± 10 | ,17[c] |
| isopropyl | | | | | ,80 |
| ethyl | 3,8 ± 0,5 | 16 ± 2 | 10 ± 2 | 43 ± 10 | ,17 |
| n-propyl | | | | | ,17 |
| isobutyl | 3,5 ± 1 | 15 ± 5 | 9 ± 2 | 38 ± 10 | ,23 |
| neopentyl | | | | | ,40 |
| cyclopropyl | | | | | ≤ ,05 |
| cyclobutyl | 5,3 ± 0,5 | 22 ± 2 | 16 ± 2 | 66 ± 10 | ,25 |
| cyclopentyl | | | | | ,40 |
| cyclohexyl | | | | | ,80 ± 10 |

a x = fraction present as the high-temperature, 'base-off' form (C) at 25°.

b Value determined at 20°C(ref 21)

c Compare x = 0,13 - 0,15 determined at 20°C(ref 21)

drawn. The spectra of cyanocobalamin in water and in 1M imidazole are identical.

Thus it appears that the change of conformation from 'normal' to 'cobalamin' (and the change of ligand from imidazole to benzimidazole) changes the position of the $\alpha, \beta$ and $\gamma$ bands by $\leq$ 2nm for both the methyl and the cyanide corrinoid complexes. On the other hand the change of ligand from $H_2O$ to imidazole as in aquocyanocobinamide and in the imidazole adduct of cyanocobinamide (with no change in conformation of the corrin ring) results in a shift of ~25nm in the $\alpha\beta$ bands and of ~10nm in the $\gamma$ band.

5.2.3 Independence of conformation and electronic effects

These results show that changes in the conformation of the corrin ring should not cause more than slight effects on the electronic spectra of corrinoids. Additional support is provided by published work; the adducts of methylcobinamide with imidazole, 1-methylimidazole, pyridine, ammonia and ethanolamine all have very similar spectra[23,70] and for the adducts of cyanocobinamide with imidazole, pyridine and ammonia the $\gamma$ bands vary by <2nm in wavelength.[84] It therefore appears that large changes in electronic structure can occur without any change in conformation, as revealed by X-ray analysis, and that changes in conformation can occur without any significant change in electronic structure, as shown by UV-visible spectroscopy ie. changes in conformation and in electronic structure can occur independently of each other.

The electronic structure of cobalamins is determined mainly by the σdonor power of the axial ligands[58] while the conformation of the corrin ring is determined mainly by the need to reduce non-bonded contacts.[47] It seems reasonable to assume that all the corrinoids where the bulky benzimidazole base is coordinated to cobalt have the 'cobalamin' conformation and that all others have the 'normal' conformation.

## 5.3 pH-Independent equilibria of organocobalamins

### 5.3.1 Effect of temperature on the equilibria of organocobalamins

The UV-visible spectra of methyl, ethyl, isobutyl and cyclobutylcobalamin show reversible temperature-dependent changes with good isosbestic points between 20°C and about 80°C in both deionized water (pH = 6,8) and dilute sodium hydroxide (pH = 9,8) ie. the equilibria are pH-independent at pH's well above the pKa values of the alkylcobalamins. The spectra recorded for these alkylcobalamins (given in Figs. 5.4 and 5.5 for ethylcobalamin and cyclobutylcobalamin) show changes on raising the temperature which correspond to a shift in equilibrium of between a red 'base-on' form (A) at low temperature and a yellow 'base-off' form (C) at higher temperature. Cyclopropylcobalamin showed changes in its spectrum on heating similar to those shown by methylcobalamin but was not studied in detail. The spectrum of neopentylcobalamin, which has only approximately sixty per cent of the 'base-on' form present at room temperature (Fig.5.6) also shows an apparently reversible and temperature-dependent shift in equilibrium on

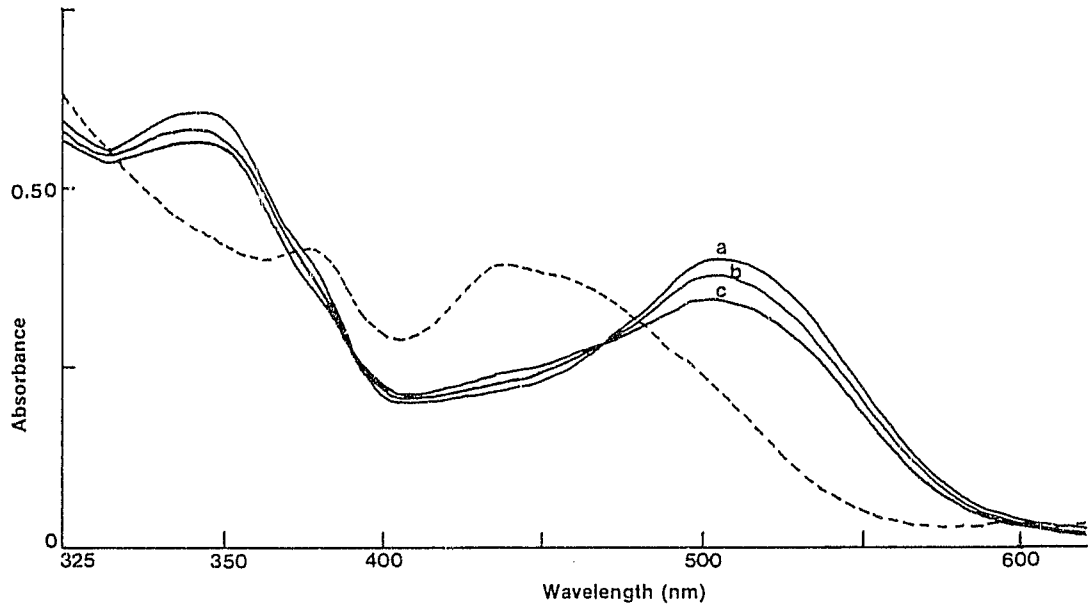



Fig.5.4 Spectra of $4,3 \times 10^{-5}$M ethylcobalamin at pH = 9,8 at (a) 20°C, (b) 48°C and (c) 68°C and (---) at pH = 1 at 25°C

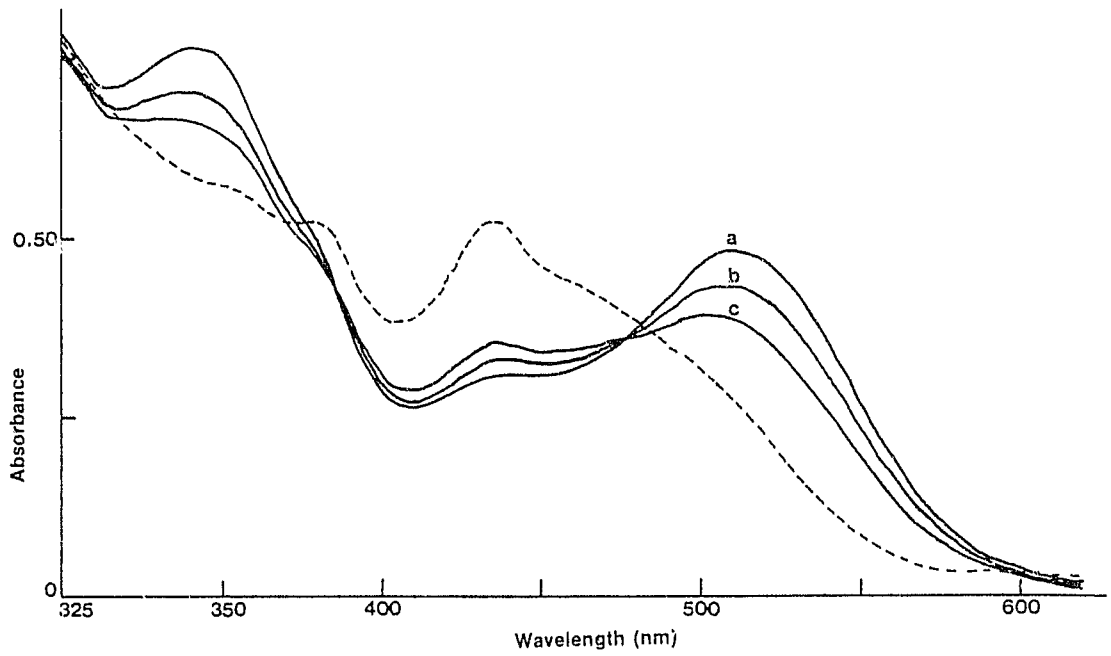



Fig.5.5 Spectra of $6,0 \times 10^{-5}$M cyclobutylcobalamin at pH = 9,8 at (a) 20°C, (b) 53,5°C and (c) 77°C and (---) at pH = 1 at 25°C.

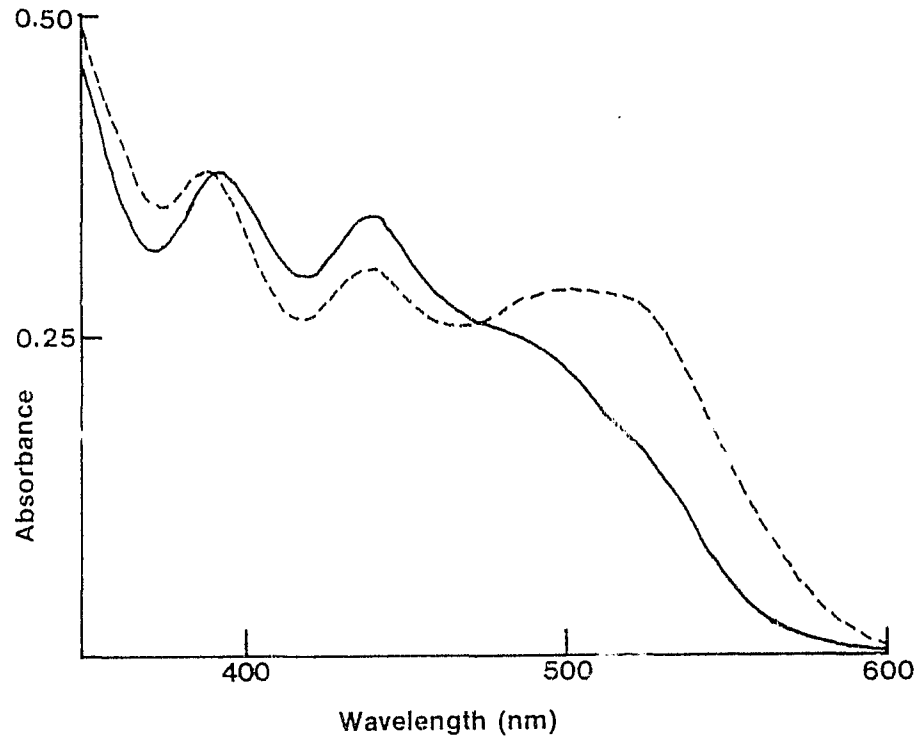



Fig.5.6 Spectrum of $3,7 \times 10^{-5}$M neopentylcobalamin at pH = 6,8 (---) and pH = 1 (—— ) at 25°C.

heating but the presence of isosbestic points is obscured by decomposition. On cooling from 20°C to 3°C the amount of 'base-on' form reversibly decreases as shown by the UV-visible spectrum. Isopropyl-, cyclopentyl- and cyclohexylcobalamin decompose even at room temperature and under nitrogen so their spectra could not be obtained at high temperature. They, however, contain high proportions of the 'base-off' form even at room temperature.

The temperature-dependent spectra of methyl, ethyl, isobutyl and cyclobutylcobalamin were used to determine values for $\Delta H$ and $\Delta S$ for the equilibrium between the 'base-on' C form and the 'base-off' A form for each alkylcobalamin and to estimate the percentage of the 'base-off' C form in neutral solution at 25°C for each alkylcobalamin. Plots of log $K_{AC}$ ($K_{AC} = [C]/[A]$) versus 1/T were made (Fig.5.7). The end-points were obtained by assuming that the spectrum of 100 per cent C is the same as that of the E form and that the spectrum of 100 per cent A is similar to that of methylcobalamin at -185°C in ethanol (for methyl- and cyclopropylcobalamin)[38] or to that of ethylcobalamin at -185°C in ethanol[38] (all others) and checked by graphical extrapolation. Values for the percentage of the C form in neutral solution (x), $\Delta H$ (from the slope of the graph of log $K_{AC}$ vs 1/T) and $\Delta S$ (from $K_{AC}$ and $\Delta H$) are given in Table 5.1. The experimental plots for the determination of $\Delta H$ are given in Fig.5.7. The estimation of the end-points for the plots in Fig.5.7 probably gives rise to inaccuracies in the values of $\Delta H$ and $\Delta S$ but the linearity of the

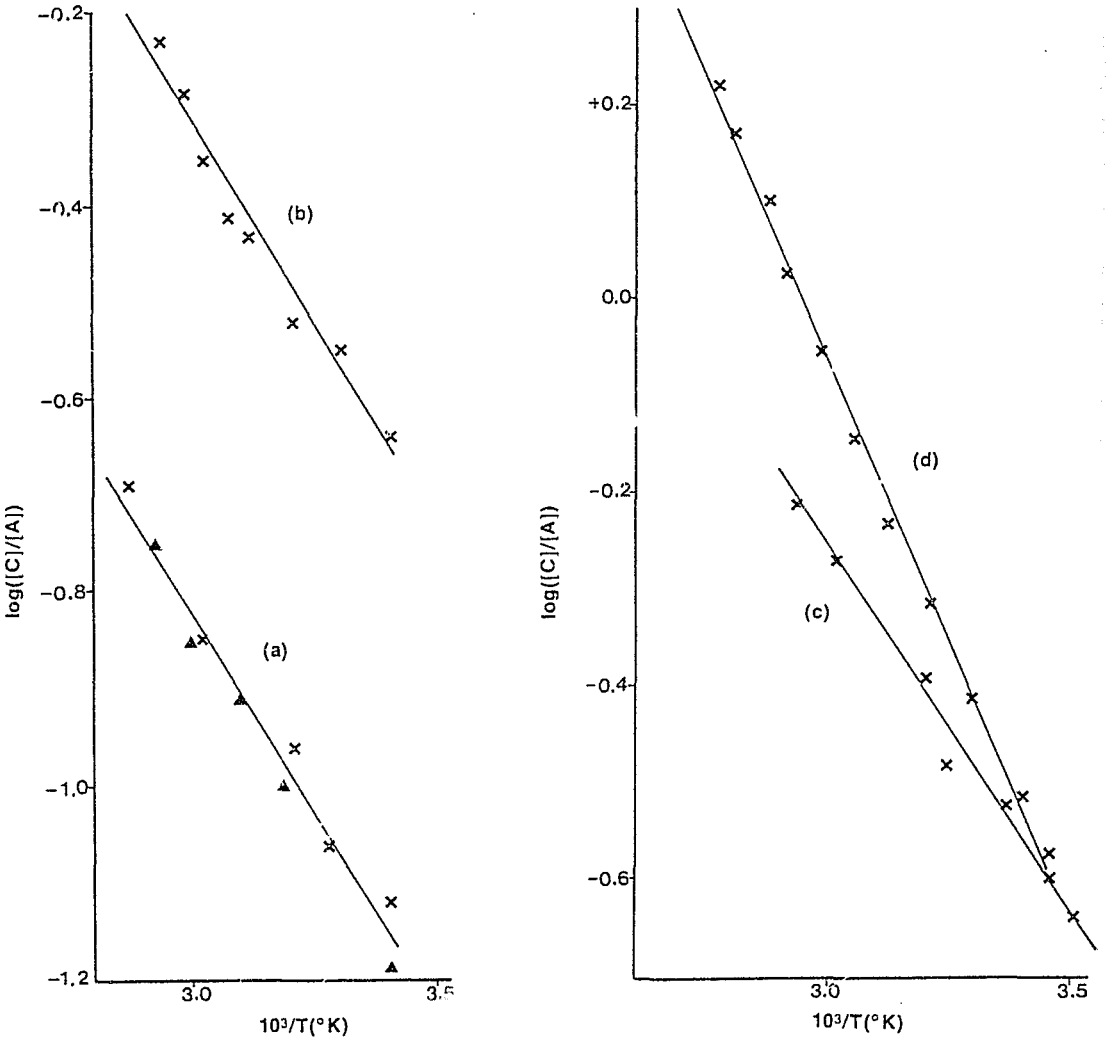



Fig.5.7 Experimental plots for the determination of ΔH for the pH-independent equilibria of (a) methylcobalamin (two separate experiments denoted by x and ▲ respectively), (b) ethylcobalamin, (c) isobutylcobalamin and (d) cyclobutylcobalamin at pH = 9,8.

of the plots suggests that the errors cannot be too great.

The percentage of C present in solutions of n-propyl-, isopropyl-, cyclopropyl-, neopentyl, cyclopentyl and cyclohexylcobalamin at room temperature and neutral pH were determined as above and are given in Table 5.1.

The proportion of C:A in neutral solution at 25°C increases as expected for increasing substitution on the α-carbon (methyl < ethyl < isopropylcobalamin), on the β-carbon (ethyl~n-propyl < isobutyl < neopentylcobalamin) and for increasing bond angle of the alicyclic ring (cyclopropyl < cyclobutyl < cyclopentyl < cyclohexylcobalamin) ie. it increases as expected with increasing steric compression. These results show a good qualitative correlation with the order observed for pKa values. However, this approach is probably valid only for isopropyl, neopentyl, cyclopentyl and cyclohexyl-cobalamin (see section 5.3.2).

5.3.2 Comparison of pKa and $K_{AC}$ for alkylcobalamins: a check on the validity of the equilibria given in Fig.1

In the previous chapter (section 4.2.2) it was shown that the species B and D are not present in detectable amounts. It is therefore reasonable to assume that the only species present for the alkylcobalamins studied are the 'base-on' form (A), the unprotonated 'base-off' form (C) and the protonated 'base-off' form (E) and that all the equilibria observed can be explained in terms of these species. The three species (A, C and E) can be linked by the two independent equilibria described by the equilibrium constants

$$Ka = \frac{[H^+]\left([A] + [C]\right)}{[E]} \text{ and } K_{AC} = [C]/[A]$$

The validity of the equilibria given in Fig.4.1 can be checked by defining a third equilibrium constant (between C and E forms).

$$K_{CE} = \frac{[C][H^+]}{E}$$

This equilibrium constant is the same as the equilibrium constant for protonation of the pendant benzimidazole base ie. $K_{CE} = K_{BZM}$ and $pK_{BZM} = -\log K_{CE}$. At the pH equal to the pKa for a particular alkylcobalamin fifty per cent of the cobalamin should be in the E form, 50x per cent in the C form and 50(1-x) per cent in the A form, where x is the fraction of form C present in neutral solution. Then

$$K_{CE} = \frac{50x/100}{50/100}\,[H^+] = x[H^+]$$

or $pK_{BZM} = pKa - \log x$.

Values of $pK_{BZM}$ were calculated for the alkylcobalamins and are listed in Table 5.2. These values should be the same for all alkylcobalamins and should be consistent with the value of $pK_{BZM} = 5,0$ found for the pendant benzimidazole base of dicyanocobalamin.[86] It can be seen quite clearly from Table 5.2 that the $K_{EC}$ values increase in the order methyl < cyclopropyl < adenosyl < ethyl ~ n-propyl < isobutyl ~ cyclobutyl < neopentyl ~ cyclopentyl ~ cyclohexyl ~ isopropyl. Only the $pK_{BZM}$ values for isopropyl-, neopentyl-, cyclopentyl- and cyclohexylcobalamin are consistent with $pK_{BZM} = 5,0$; the pKa values for methyl-,

Table 5.2 - Calculation of the pK of the Pendant BZM($pK_{Bzm}$)

| Ligand | $pK_a$ | x | $pK_{Bzm}$ |
|---|---|---|---|
| Methyl | 2,5 - 2,7 | ,05 | 3,8 - 4,0 |
| cyclopropyl | 2,9 $\pm$ 0,1 | $\leq$ ,05 | $\geq$ 4,1 |
| adenosyl | 3,3 - 3,5 | ,10 | 4,3 - 4,5 |
| ethyl | 3,87 | ,19 | 4,6 |
| n-propyl | 3,84 | ,17 | 4,6 |
| cyclobutyl | 4,16 - 4,18 | ,25 | 4,7 - 4,8 |
| isobutyl | 4,15 - 4,20 | ,23 | 4,8 |
| cyclopentyl | $\geq$ 4,5 | ,40 | $\geq$ 4,9 |
| cyclohexyl | 4,7 $\pm$ 0,2 | ,80 | 4,6 - 5,0 |
| isopropyl | $\geq$ 4,5 | ,80 | $\geq$ 4,6 |
| neopentyl | 4,7 $\pm$ 0,2 | ,40 | 4,9 - 5,3 |

$pK_{Bzm} = pK_a - \log x$,

where x = fraction present as C at 25°C

and $K_a = \frac{[H^+]([A] + [C])}{[E]}$

Values of x and $pK_a$ are taken from Table 5.1 and Table 4.4 respectively.

cyclopropyl- and adenosylcobalamin are certainly and those for ethyl, n-propyl and cyclobutyl probably out of line. For isopropyl-, neopentyl-, cyclopentyl- and cyclohexylcobalamin the three species A, C and E are adequate to describe the equilibria observed. For the other alkylcobalamins one of the assumptions made in the calculation of x must be wrong. The wrong assumption, as shown in the next section, is that there are only three species.

5.3.3 Comparison of the spectra of the unprotonated 'base-off' C form and the protonated 'base-off' E form

For any particular alkylcobalamin the C and E forms should have spectra (above 300nm) identical to each other and to the G form of the corresponding alkylcobinamide if the spectrum is not affected by weak interactions between the benzimidazole side-chain and the corrin ring. It is impossible to compare the spectra for the unprotonated 'base-off' C forms and the protonated 'base-off' E forms because the C form is never obtained in the pure state. At the one end of the range of alkylcobalamins is methylcobalamin which is stable enough in solution to be heated to 90°C but still has a large proportion of A present at this temperature and at the other is isopropylcobalamin where eighty per cent C is present at room temperature but decomposition occurs even at this temperature. Because of this a comparison was made of the changes in spectra observed on heating the alkylcobalamins and on their acidification.

The spectrum of ethylcobalamin in neutral or basic solution has a shoulder at 375nm while the spectrum in acidic solution has

an obvious band at 380nm. Interconversion between the acidic and basic forms gives rise to isosbestic points at ~ 380nm and ~ 480nm (Fig.5.4). If ethylcobalamin in basic solution is heated from 20°C to 68°C the shoulder at 380nm gradually disappears and isosbestic points are seen at 390nm and 470nm (Fig.5.4). The spectra of ethylcobinamide in neutral and acidic solution are identical to each other and to that of ethylcobalamin in acid solution.

The spectrum of isobutylcobalamin in neutral or basic solution has a shoulder at ~ 378nm while the spectrum in acidic solution has an obvious band at 384,5nm. Interconversion between the acidic and basic forms gives rise to isosbestic points at ~ 380nm and ~ 483nm. If isobutylcobalamin in neutral solution is heated from 20°C to 67°C the shoulder at ~ 378nm gradually disappears and isosbestic points are seen at 390nm and 482nm. The spectrum of isobutylcobinamide is very similar to that of isobutylcobalamin at acid pH, apart from a slight difference in the relative intensities of the bands at 440nm and 460nm.

The spectrum of cyclobutylcobalamin in neutral or basic solution has a shoulder at ~ 380nm while the spectrum at acidic pH has an obvious band at 378nm. Interconversion between the acidic and basic forms gives rise to isosbestic points at ~ 380nm and ~ 483nm (Fig.5.6). If [illegible] cobalamin in basic solution is heated from 20°C - 70°C the shoulder at ~ 380nm gradually disappears

and isosbestic points are seen at 385nm and 477nm (Fig.5.5). The spectrum of cyclobutylcobinamide is very similar to that of cyclobutylcobalamin at acid pH, apart from a slight difference in the relative intensities of the bands at 440nm and 460nm (Fig.4.5).

The isosbestic points for the interconversion of the acidic and basic forms of methylcobalamin occur at ~386nm and ~490nm and those for the temperature-dependent equilibrium at basic pH at ~384nm and ~472nm. It cannot be said conclusively whether there are changes in the 370-380nm region of the methylc spectrum analogous to those for ethyl, isobutyl and cyclobutylcobalamin because of the small overall change in absorbance with temperature. The spectra of methylcobalamin and methylcobinamide at acidic pH are identical.

Neutral and basic solutions of isopropyl-, neopentyl-, cyclopentyl- and cyclohexylcobalamin, which contain forty to eighty per cent C, have prominent bands in the 370-390nm region. In spite of the instability of these cobalamins, it can be seen that the five-coordinate, protonated, 'base-off' (E) form and the five-coordinate, unprotonated, 'base-off' (C) form have very similar (probably identical) spectra. The spectra of isopropyl- and cyclohexylcobalamin in acid solution are very similar to those of isopropyl or cyclohexylcobinamide in acidic or neutral solution. The spectrum of neopentylcobinamide in acid and neutral solution is identical to that of neopentylcobalamin in acid solution in the 370-390nm region but there are slight

differences in the relative intensities of the absorbance bands at 440 and 460nm. The spectrum of cyclopentylcobinamide was not checked.

It appears that for methyl, ethyl, isobutyl and cyclobutylcobalamin that the spectrum (in the 370-390nm region) of the unprotonated 'base-off' form (C) is not the same as that of the protonated base-off form (E). This variant form of C will be referred to as $C_{BZM}$. It probably differs from C because of the presence of some weak interaction of the benzimidazole side-chain with the corrin ring. The very small difference in spectrum between C and $C_{BZM}$ means that the difference between C and $C_{BZM}$ is not caused by a change in coordination number. For isopropyl, neopentyl, cyclopentyl and cyclohexylcobalamin the spectra of the C and E forms are apparently identical and there is no need to postulate $C_{BZM}$ form. The forms C and $C_{BZM}$ can be characterized by the presence (C) or absence ($C_{BZM}$) of a band at 370-390nm. It appears that C is the predominant 'base-off' form of cobalamin at neutral pH for isopropyl, neopentyl, cyclopentyl and cyclohexylcobalamin and that $C_{BZM}$ is the predominant form for all the others at high temperatures and probably also at room temperature.

5.3.4 Nature of $C_{BZM}$

As shown in section 5.3.3 an alternative form of C (ie. $C_{BZM}$) exists, is distinguished from C by its UV-visible spectrum and probably differs from C in the interaction of the unprotonated

benzimidazole side-chain with the corrin ring.

Further evidence for a species additional to forms A, B and C (shown in Fig.4.1) in neutral solution is provided by the results of Brown et al[87] using temperature-jump spectroscopy. They studied the kinetics of the 'base-on'/'base-off' equilibrium of methylcobalamin at 5°C and found two pH-independent relaxations over the pH range 0,46 - 7,42. This corresponds to three types of complex, which they assumed to be A, B and C (III, II and IV in their terminology), connected by pH-independent equilibria in addition to the protonated 'base-off' form E (I in their terminology). The assumption that the three complexes in neutral solution are A, B and C leads to some rather odd conclusions such as that the 'base-on' species of methylcobalamin is only approximately thirty per cent coordinated to benzimidazole at 5°C and that the pKa for protonation of the 'base-off' benzimidazole of methylcobalamin must be 4,0 or less (compare the calculated value for methylcobalamin in Table 5.2).

The UV-visible spectrum of adenosylcobalamin in basic solution shows the same type of changes, in particular the disappearance of the band at 376nm, as described for methyl, ethyl, isobutyl and cyclobutylcobalamin.[38] However, the NMR studies of Cockle et al[88,89] on adenosylcobalamin suggest that the benzimidazole base remains close to $C_{20}$ of the corrin ring when adenosylcobalamin is heated in $D_2O$. This indicates that in the $C_{BZM}$ forms there must be some interaction between the

benzimidazole side-chain and the corrin ring. Cockle et al[89] concluded that the benzimidazole base is coordinated (via N7), to the cobalt by a very long bond. This does not satisfactorily explain why the UV-visible spectrum shows no evidence for interaction of the benzimidazole base with the cobalt (the spectra of C and of $C_{BZM}$ are very similar).

The suggestion that the changes occurring in the UV-visible spectra of organocobalamins on heating are due to changes in conformation[88,89,90] is unlikely to be true as it has been shown (section 5.2) that conformational changes have very little effect on the UV-visible spectra of corrinoids.

It seems more likely that the benzimidazole side-chain is held close to $C_{20}$ by interactions (eg. hydrophobic, hydrogen-bonding) between the benzimidazole side-chain and the corrin ring than that benzimidazole is coordinated to cobalt. Benzimidazole, when not protonated, is fairly hydrophobic and could easily interact with a hydrophobic part of the corrin ring even if it is not coordinated to cobalt. Possible positions for the uncoordinated benzimidazole group are suggested by a study of models. These are the hydrophobic clefts either between $C_{20}$ and $C_{30}$ and $C_{41}$ (a position similar to that occupied by benzimidazole coordinated to cobalt) or between $C_{20}$ and $C_{48}$. Either of these positions would explain the effect of benzimidazole on the $C_{20}$ methyl resonance. We therefore suggest a structure for $C_{BZM}$ in which the cobalt atom is essentially five-coordinate (in accordance

with the evidence from UV-visible spectra) and the benzimidazole base is held, mainly by hydrophobic interactions, in one or both of the hydrophobic clefts mentioned above (which would explain the NMR results). There is also a possibility of additional charge transfer interaction with the conjugated part of the corrin ring or of weak coulombic interaction with the residual positive charge on the cobalt.

5.4 Effect of the organoligand on the pH-independent equilibrium between A and C

The ratio of five-coordinate, unprotonated, 'base-off' (C) forms to six-coordinate, 'base-on' (A) forms increases in the order cyclopropyl $\leq$ methyl $<$ adenosyl $<$ ethyl $\sim$ n-propyl $<$ isobutyl $<$ cyclobutyl $<$ neopentyl $\sim$ cyclopentyl $<$ isopropyl $\sim$ cyclohexyl (Table 5.1) and shows a good parallel with the pKa's and spectral parameters discussed in Chapter 4. The observed order is consistent with the greater stability of five-coordinate relative to six-coordinate forms for sterically hindered organocobalamins. The amount of the C form relative to the $C'_{BZM}$ form (as shown by the discrepancy between pKa and $pK_{BZM}$ in Table 5.2) also increases with increasing steric compression caused by the organoligand.

5.5 Summary

It is shown that changes in conformation and in electronic structure for corrinoids can occur independently of each other and that changes in conformation have little effect on the UV-visible spectra of corrinoids. The experimental data show that there are four types of complexes in solution for cobalamins having a

tetrahedral carbon atom coordinated to cobalt viz. A, C, $C_{BZM}$ and E (see Fig.4.1) where A is the six-coordinate, 'base-on' form; C and $C_{BZM}$ are five-coordinate, 'base-off' forms in neutral solution; and E is the five-coordinate, 'base-off' form in acid solution. It is shown that the enthalpy and entropy changes on the conversion of the A to C forms are as expected for the conversion of the six- to five-coordinate forms (cf. the values for cobinamides[21]). It is suggested that in the C form the benzimidazole side-chain does not interact with the corrin ligand but in the $C_{BZM}$ form there is hydrophobic bonding between the benzimidazole side-chain and the corrin ligand.

# CHAPTER 6 - HOMOLYTIC FISSION OF NEOPENTYLCOBALAMIN

## 6.1 Introduction

The spectra and equilibria of neopentylcobalamin indicate that there is considerable steric interaction between the neopentyl ligand and the rest of the cobalamin molecule, giving rise to a long, weak Co-C bond in neopentylcobalamin. Neopentylcobalamin, like isopropylcobalamin and cyclohexylcobalamin, readily decomposes in air at room temperature but, since it has no β-hydrogen, it cannot decompose by β-elimination. The most likely mechanism for decomposition of neopentylcobalamin is homolytic fission, via the equilibrium

$$Co\text{-}R \rightleftharpoons Co(II) + R\cdot$$

If neopentylcobalamin decomposes by homolytic fission it would be useful as a model for the homolytic fission of adenosylcobalamin during the isomerase enzyme reactions.

Since neopentylcobalamin is stable under nitrogen at room temperature and no bands due to $B_{12r}$ are observable in its spectrum the proposed equilibrium must be shifted to the left under these conditions and it is necessary to find evidence that the equilibrium does occur. In order to do this the effect on neopentylcobalamin of reagents which are known to promote homolytic fission of organocobalamins will be studied and the reactions of

neopentylcobalamin will be compared to the homolytic (mainly photolytic) reactions of other organocobalamins.

6.2 Homolysis of the Co-C bond in alkylcobalamins

In this section the homolytic reactions (mainly initiated by photolysis) will be described as a basis for the comparison of the reactions of neopentylcobalamin. Simple primary organocobalamins eg. methyl and adenosylcobalamin do not undergo homolytic reactions in the dark and at room temperature.[5f]

6.2.1 Photolysis of organocobalamins

The first step in the photolysis of organocobalamins is the homolytic cleavage of the Co-C bond to give $B_{12r}$ and the free radical.

$$Co\text{-}R \longrightarrow Co(II) + R\cdot$$ [91,92]

Flash photolytic experiments show that the Co-C bond in methyl and adenosylcobalamin is easily broken giving $B_{12r}$ and the methyl or adenosyl radical.[92,93] The adenosyl and the ethyl radical have been observed in spin-trapping experiments on ethylcobalamin.[94]

The photolysis of methylcobalamin proceeds apparently very slowly, giving $B_{12r}$,[95,96] methane and ethane[97] under anaerobic conditions. This is because the recombination of $B_{12r}$ and the methyl free radical occurs very rapidly ($k \sim 1,5 \times 10^9$ $l mol^{-1} s^{-1}$).[92] The photolysis of methylcobalamin is accelerated in the presence of oxygen, giving $B_{12a}$[95] and formaldehyde.[97] In the presence of oxygen the methyl radical is converted to the methylperoxy radical and then to formaldehyde.[5f] In the absence of oxygen the

methyl radical can abstract a hydrogen atom from the corrin ring or the solvent (to give methane), abstract a methyl group from the corrin ring or dimerize (to give ethane) or react with the corrin ring to give modified corrinoids.[5f]

Hydrogen-donors such as alcohols and thiols[96] also accelerate the rate of photolysis of methylcobalamin. These reagents can be placed in the order of their effect on the acceleration of photolysis (assuming for each that the rate of photolysis is proportional to concentration) oxygen $\gg$ thiols $\gg$ alcohols $>$ no added reagent.[5f] All of these reagents presumably show their effect by their removal of the methyl radical. The effect of alcohols on the rate of photolysis increases with the ease of abstraction of a hydrogen atom from the $\alpha$-position of the alcohol ie. isopropanol $>$ n-propanol $>$ ethanol $>$ methanol.[96] It was shown for the photolysis of methylcobalamin in the presence of isopropanol that pinacol is formed;[96] presumably the methyl radical abstracts a hydrogen atom from the isopropanol giving methane (this was not checked) and the $\alpha$-hydroxypropyl radical which dimerizes to give pinacol (identified in the reaction mixture.[96] The photolysis of methylcobalamin in the presence of the thiols cysteine and homocysteine produces S-methylcysteine[98] and methionine[96] respectively.

For adenosylcobalamin the rates of photolysis in the presence and absence of oxygen are very similar.[95] Under anaerobic conditions adenosylcobalamin is rapidly photolysed

(much more rapidly than methylcobalamin) to give $B_{12r}$[95] and 8,5'-cyclic adenosine (from cyclization of the adenosyl free radical on to the imidazole ring of the adenine group.[99] In the presence of air, $B_{12a}$[5f] and a mixture of 8,5'-cyclic adenosine and adenosine-5-aldehyde is formed.[100] For the photolysis of adenosylcobalamin the rate determining step is the breaking of the Co-C bond rather than the subsequent reaction of the free radical.[5f] Photolysis of an analogue of adenosylcobalamin, 2,3'-isopropylidene-5'-deoxyuridinylcobalamin, in the absence of air gives $B_{12a}$ and a cyclic nucleotide product. Presumably the first step is homolysis of the Co-C bond to give $B_{12r}$ and a free radical followed by cyclization and reduction of the radical by $B_{12r}$.[101]

In isopropanol flash photolysis of adenosylcobalamin produced $B_{12r}$ which in a subsequent dark reaction was converted to $B_{12s}$.[93] It was postulated that isopropanol would efficiently scavenge the adenosyl radical, producing the strongly reducing α-hydroxypropyl radical which would reduce $B_{12r}$ to $B_{12s}$ and itself be oxidized to give acetone.[93] Adenosine has been identified after photolysis of adenosylcobalamin in isopropanal[102] which fits in with the above postulate. In the presence of thiols, the photolysis of adenosylcobalamin generally gives rise to adenosine (presumably by abstraction of a hydrogen atom from the thiol by the radical),[102, 103, 104] except for homocysteine which gives S-adenosylhomocysteine.[98] The valency of the cobalt was not specified.

Photolysis of ethylcobalamin and higher alkylcobalamins is similar to that of methylcobalamin. Anaerobic photolysis is faster than for methylcobalamin[105] and gives as products mainly alkenes.[106] The reaction is accelerated by air (giving $B_{12a}$[97, 106] and aliphatic aldehydes[97]) and alcohols such as isopropanol (giving $B_{12r}$[105]). One interesting difference from the photolysis of methylcobalamin is the appearance of $B_{12s}$, as well as $B_{12r}$ on the photolysis in the absence of air of alkylcobalamins.[105] This has been ascribed to the reduction of $B_{12r}$ to $B_{12s}$ by the alkyl radical with the concomitant formation of an alkene.[105] The formation of $B_{12s}$ could also be due to β-elimination as described in Chapter 7.

For methylcobalamin photolysis is reversible in the absence of added reagents. For adenosylcobalamin and higher alkylcobalamins photolysis is not reversible since the free radical is able to terminate by other pathways (cyclisation in adenosylcobalamin, oxidation to the olefin in the higher alkylcobalamins).[5f]

6.2.2 Thermolysis of methylcobalamin

Thermolysis of methylcobalamin has been less extensively studied than photolysis but is presumably also a homolytic reaction giving rise to $B_{12r}$ and the methyl free radical. Evidence for the free-radical nature of this reaction is the similarity of the organic products to those obtained from photolysis of methylcobalamin and the isomerization of D and L isomers on heating

or photolysis under nitrogen. The isomerization reaction presumably involves the formation of $B_{12r}$ and methyl radicals and their recombination.[5n]

Neopentylcobalamin is expected to undergo homolysis in the dark and at room temperature in a way similar to methylcobalamin in the presence of light ie. the reaction Co-R $\rightleftharpoons$ Co(II) + R· should be reversible for neopentylcobalamin under nitrogen and little or no net formation of $B_{12r}$ should be observed. By analogy to the reactions of methylcobalamin and adenosylcobalamin we expect

(a) the decomposition of neopentylcobalamin to be accelerated by oxygen, thiols, alcohols, adenine and other heterocycles

(b) the order of reactivity to be oxygen $\gg$ thiols $\gg$ alcohols $>$ no added reagent

(c) the formation of neopentane in the presence of thiols and alcohols.

6.3 Experimental procedures

Reactions of neopentylcobalamin were studied at acidic pH where neopentylcobalamin is entirely in the protonated 'base-off' form (section 4.2.2) and at neutral pH where neopentylcobalamin is sixty per cent in the 'base-on' form and forty per cent in the unprotonated 'base-off' form (section 5.3.1). The reactions of neopentylcobinamide, which is in the 'five-coordinate' form at

all pH's, were also studied and compared to those of neopentylcobalamin. The decomposition of neopentylcobalamin in the presence and absence of air and in the presence of reagents which should react with free radicals (ie. alcohols, thiols and imidazole) was followed using UV-visible spectroscopy. The cobalamin products were identified by their characteristic UV-visible spectra. Gas chromatography and thin layer chromatography on silica gel (Merck) were used to identify organic products for some of the reactions of neopentylcobalamin.

The preparation of neopentylcobalamin is described in section 3.5. This method of preparation gave rise to solutions of neopentylcobalamin of concentration varying from 1 - 5 x $10^{-4}$M in 5 x $10^{-4}$M sulphuric acid. These solutions were diluted as required and used for all experiments on neopentylcobalamin. Neopentylcobinamide was prepared as described in section 3.6.4 in the spectrophotometer cell. The reagents used are listed in section 2.1.4.

6.3.1 Reactions followed by UV-visible spectroscopy

The products and rates of decomposition of neopentylcobalamin and neopentylcobinamide were studied using UV-visible spectroscopy, scanning the range 300-650nm. The concentration of cobalamin or cobinamide in the spectrophotometer cell was approximately 3 x $10^{-5}$M. For reactions at acidic pH (pH~1) neopentylcobalamin or neopentylcobinamide was diluted with sulphuric acid and for reactions at neutral pH with sodium-potassium phosphate buffer of

the required pH (section 2.1.3). For the reactions with imidazole no phosphate buffer was used and the imidazole was self-buffering. All reactions, unless otherwise specified, were performed under anaerobic conditions. The reaction mixtures were deoxygenated by passing a stream of nitrogen through them for five minutes before mixing and during mixing and the reactions followed in spectrophotometer cells closed with a rubber diaphragm seal (Suba-Seal). The reagents studied were the alcohols, isopropanol and n-propanol; the thiols, cysteine and thioglycollate; and imidazole. Reactions were also studied in air-saturated solutions (where a brisk stream of air was passed through the reaction mixture for ten minutes and the cell then allowed to stand open to the atmosphere) in the absence of other reagents. The exact concentrations and pH values for each individual experiment are given in Table 6.1, together with the major products and the rates of reaction. The rates of product formation were followed at 350nm for $B_{12a}$ and 473nm for $B_{12r}$. Photolysis by white light (section 2.2.7) was used to complete the decomposition and obtain the final spectrum for determining the rate of decomposition in the case of the slower reactions.

6.3.2 Reactions followed by gas chromatography

The reactions of neopentylcobalamin in the absence of reagents (at 60°C) and with isopropanol and thioglycollate were followed by gas chromatography. The reaction mixtures to be analysed were contained in a 500ml flask, closed with a rubber

Table 6.1 - Reactions of Neopentylcobalamin and Neopentylcobinamide

| Complex | pH | Reagent[a] | Main Product | Rate of Reaction[b] |
|---|---|---|---|---|
| Neopentyl-cobalamin (base-on) | 6,8 | none | - | - (over 4 hours) |
| | 6,8 | none[c] | $B_{12r}$ | 23%, 24% |
| | 6,8 | air-saturated | $B_{12a}$ | 55, 60, 60 minutes |
| | 6,8 | 0,028 M cysteine | $B_{12r}$ | 3,5 hours (14%) |
| | 6,7 | 0,04 M thioglycollate | $B_{12r}$ | 20 minutes |
| | 7,2 | 3,3M isopropanol | $B_{12r}$ | 60 minutes (50%) |
| | 6,8 | 1,3M isopropanol | $B_{12r}$ | 30% |
| | 7,1 | 3,3M n-propanol | $B_{12r}$ | 15% |
| | 9,2 | 1,3M imidazole | Co(III)[d] | 5 minutes[e] |
| | | | - | |
| neopentyl-cobalamin (base-off) | 1,0 | none | - | - |
| | 1,0 | air saturated | - | - |
| | 2,2 | 0,1M cysteine | - | - |
| | 1,3 | 1,3M isopropanol | - | - |
| neopentyl-cobinamide | 6,7 | none | - | - |
| | 6,7 | air saturated | - | - |
| | 6,7 | 3,3M isopropanol | - | - |
| | 8,4 | 6M imidazole | Co(III)[f] | 30%[e] |

a All solutions contained approximately $3 \times 10^{-5}$M Co and were studied under $N_2$ (unless otherwise stated) at 25°C.

b Where decomposition is observed, the rate is given as either (i) the half-time of reaction or (ii) the percentage decomposed in one hour " —— " denotes no detectable ( 2%) decomposition in one hour.

c Temperature = 55°C. d Imidazole-cobalamin. e See text.

f Bis(imidazole) cobinamide.

diaphragm seal (Suba-Seal). Solutions consisted of

(A) 400ml of $6 \times 10^{-5}$M neopentylcobalamin, 4,9M isopropanol and phosphate buffer pH = 7,1;

(B) 300ml of $6 \times 10^{-5}$M neopentylcobalamin, 7,8M isopropanol and phosphate buffer pH = 7,3;

(C) 300ml of $1,7 \times 10^{-4}$M neopentylcobalamin, 0,15M thioglycollate and phosphate buffer pH = 5,6;

(D) 300ml of $1,7 \times 10^{-4}$M neopentylcobalamin, 1,2M thioglycollate and phosphate buffer pH = 5,6;

(E) 300ml of $6 \times 10^{-5}$M neopentylcobalamin and phosphate buffer pH = 7,2;

(F) 400ml of 4,9M isopropanol and phosphate buffer pH = 7,1 and

(G) 300ml of 0,15M thioglycollate and phosphate buffer pH = 5,6.

For solutions A, C, E, F and G samples of the gas phase were withdrawn and analysed every thirty to sixty minutes. For solutions B and D samples of the gas phase were analysed after completion of the reaction (as shown by the presence of $B_{12r}$). Solution E was thermostatted at 60°C, the other solutions were kept at room temperature. All the solutions were thoroughly deoxygenated before mixing by evacuation using a vacuum pump for twenty minutes, followed by passing nitrogen through the solution for twenty minutes.

Gas chromatography was performed using column 1 (section

2.2.9). The temperatures and carrier gas pressure used are given in Table 6.2. Peaks were identified by comparing their retention times to those of authentic compounds. Retention times of peaks varied from day to day due to slight changes in operating conditions.

## 6.4 Results and discussion

For all reactions under nitrogen (except with imidazole) the major product was identified as $B_{12r}$ from the wavelengths of the main absorption bands and the conversion to the spectrum of $B_{12r}$ to $B_{12a}$ on the admission of air.[5q] The product spectra show a band at 473nm at pH = 6 - 7 and at 470nm at pH = 1 as expected for 'base-on' and 'base-off' $B_{12r}$ respectively.[5b] During the course of the reactions isosbestic points could be observed, though these were to some extent obscured by the formation of products other than $B_{12r}$ during the reaction. The absorbance at 473nm or 470nm of the final spectrum (at the completion of the reaction) showed the presence of 60 - 100 per cent $B_{12r}$.

The admission of air to these reaction mixtures gave rise to spectra which showed, in addition to $B_{12a}$, the presence of varying amounts of 'stable yellow corrinoids', characterized by the presence of a band at 465nm which was not affected by light and air and was shifted to 485nm on the addition of cyanide.[5d] It was difficult to decide whether the 'stable yellow corrinoids' were present before the admission of air or only formed after air

Table 6.2 - Retention Times and Identification of Peaks seen in Gas Chromatographic Reactions[a]

Isopropanol reaction mixtures

| | Retention time of peak | Identification of peak |
|---|---|---|
| Solution A | 1,4 minutes | neopentane (authentic neopentane 1,4 minutes) |
| | 2,1 minutes | isopropanol (authentic isopropanol 2,1 minutes) |
| Solution B[b] | 1,6 minutes | neopentane (authentic neopentane 1,6 minutes) |
| | 2,2 minutes | isopropanol (authentic isopropanol 2,2 minutes) |
| Solution F blank | 2,1 minutes | isopropanol (authentic isopropanol 2,1 minutes) |

Thioglycollate reaction mixture

| | Retention time of peak | Identification of peak |
|---|---|---|
| Solution C[c] | 1,3 minutes | neopentane (authentic neopentane 1,3 minutes) |
| | 6 - 18 minutes (many peaks) | not identified[d] |
| Solution D[c] | 1,3 minutes | neopentane (authentic neopentane 1,3 minutes) |
| | 6 - 18 minutes (many peaks) | not identified[d] |
| Solution G[d] blank | 6 - 18 minutes (many peaks) | not identified[d] |

Solution heated to 60°C

| | Retention time of peak | Identification of peak |
|---|---|---|
| Solution E | 1,6 minutes | neopentane (authentic neopentane 1,6 minutes) |

a Temperature = 60°C, He pressure = 5psi unless otherwise indicated

b Temperature = 40°C, He pressure = 5psi

c Temperature increased to 200° C after four minutes.

d See text.

was admitted because of the overlap of the band at ~465nm due to the 'stable yellow corrinoid' with the band at 473nm due to $B_{12r}$. Some side-reactions must have occurred under nitrogen since the amount of $B_{12r}$, seen in the final spectrum, was less than that expected from the complete decomposition of neopentylcobalamin and because of the absence of good isosbestic points. The presence of these stable yellow corrinoids complicates the interpretation of results for the reactions of neopentylcobalamin under nitrogen.

For reactions of neopentylcobalamin in air the product was identified, from its UV-visible spectrum, as $B_{12a}$. For reactions of neopentylcobalamin in the presence of imidazole the product was identified as imidazolecobalamin by comparison with the spectrum obtained by reaction of $B_{12a}$ with imidazole.

The reactions of neopentylcobalamin and neopentylcobinamide are summarized in Table 6.1.

For reactions under nitrogen the organic product was shown by gas chromatography, to be neopentane (Table 6.2).

6.4.1 Reactions under nitrogen in the absence of added reagents

Neopentylcobalamin in neutral solution (phosphate buffer or acetate buffer) shows $\leq$ two per cent decomposition in four hours and in acid (pH = 1,0) solution shows $\leq$ two per cent decomposition in twenty hours in the absence of air. Neopentylcobinamide shows $\leq$ two per cent decomposition in one hour (it was not checked for a longer period) in neutral solution in the absence of air.

On heating under nitrogen to 55°C neopentylcobalamin in neutral solution slowly decomposed to give $B_{12r}$ (twenty-four per cent in one hour)(Table 6.1). There is probably some reaction with the corrin ring since stable yellow corrinoids were observed (at ~465nm) on admitting air to the reaction mixture. Gas chromatography of the gas phase of a reaction mixture of neopentylcobalamin which was heated at 60°C at pH = 7,2 showed the presence of neopentane (Table 6.2), presumably formed by hydrogen abstraction by the neopentyl radical from the corrin ring and/or the side-chains.

6.4.2 Reactions in the presence of air

Neopentylcobalamin in acid solution (pH = 1,0) and neopentylcobinamide in neutral solution are stable ( ≤ two per cent decomposition) for at least one hour in the presence of air. Neopentylcobalamin at neutral pH in the presence of air decomposes to give $B_{12a}$ with a half-life of sixty minutes (Table 6.1). Good isosbestic points are observed for this reaction. The decomposition of neopentylcobalamin at neutral pH in the presence of air is much faster than under nitrogen and is much faster in the 'base-on' form than for the 'base-off' form, either cobinamide or cobalamin. The neopentyl radical presumably reacts with oxygen to give an aldehyde. No stable yellow corrinoids are observed and there is presumably little or no radical attack on the corrin ring.

6.4.3 Reactions in the presence of alcohols

Neopentylcobalamin in acid solution and neopentylcobinamide were stable for at least one hour in the presence of 1,3 and 3,2M isopropanol respectively. At neutral pH neopentylcobalamin was decomposed to give $B_{12r}$ at a rate of thirty per cent in one hour for 1,3M . propanol and fifty per cent in one hour for 3,3M isopropanol. n-Propanol (3,3M) at neutral pH decomposed neopentylcobalamin to give $B_{12r}$ at a rate of fifteen per cent in one hour (Table 6.1). Reasonable isosbestic points were observed for these reactions.

The slower rate of decomposition by n-propanol than by isopropanol fits in with the fact that it is easier to abstract a hydrogen atom from isopropanol than from n-propanol.[96] Isopropanol and n-propanol accelerate the decomposition of neopentylcobalamin (compared to the reaction under $N_2$) as expected if a neopentyl radical is formed.

Stable yellow corrinoids (at 465nm) were observed on admitting air to the reaction mixture. These were probably present before the admission of air because the $B_{12r}$ spectrum showed increased absorbance about 450nm compared to the peak at 473nm and the amount of $B_{12r}$ observed (from the peak at 473nm) was only about sixty per cent of that expected for complete conversion of neopentylcobalamin to $B_{12r}$.

In order to check that $B_{12r}$ is the primary product of the reaction the effect of isopropanol on $B_{12a}$ and $B_{12s}$ (the possible

alternative products) was determined under conditions similar to those for the reactions of neopentylcobalamin in isopropanol. $B_{12a}$ showed no reaction in the presence of 1M isopropanol at pH = 6,6. $B_{12s}$, however, was rapidly (reaction complete in less than three minutes) converted to $B_{12r}$ in the presence of 1,0M isopropanol at neutral pH. This means that $B_{12s}$ could conceivably be formed and not be detected during these experiments. Isopropanol also prevented the reduction of $B_{12r}$ to $B_{12s}$ by sodium borohydride but this may be due to the known decomposition of sodium borohydride by alcohols.[49] Thus, the observation of $B_{12r}$ is evidence in favour of, but not conclusive proof for, $B_{12r}$ being the primary product of photolysis.

Gas chromatography of samples taken at thirty to sixty minute intervals from the gas phase above the isopropanol reaction mixture (section 6.3.2) showed a peak with the same retention time as neopentane, which was not present at the start of the reaction, increased in height over seven hours and then levelled off. This was the only peak present which did not also appear in the GC trace of the gas phase above the blank reaction (Table 6.2). The neopentane observed presumably arises from abstraction of a hydrogen atom from isopropanol to give the α-hydroxypropyl radical.

The α-hydroxypropyl radical could terminate by:

(a) an oxidation-reduction reaction converting the α-hydroxypropyl radical and $B_{12r}$ to acetone and $B_{12s}$, as occurs in the flash photolysis of adenosylcobalamin;[93]

(b) dimerization of the α-hydroxypropyl radical to give pinacol as occurs in the photolysis of methylcobalamins.[96]

(c) attack on the corrin ring to give stable yellow corrinoids (Section 1.6).

No acetone was observed on the GC trace of the gas phase over the isopropanol reaction mixture. Attempts to detect pinacol by extracting the isopropanol reaction mixture with ether and thin-layer chromatography (on silica) of the concentrated ether extract were unsuccessful. Two spots ($R_f$ = 0,08 and $R_f$ = 0,77 in chloroform/methanol 10:1) were observed, neither of which corresponded to pinacol ($R_f$ = 0,60 in chloroform/methanol 10:1). Under these conditions formation of acetone and of pinacol are not the major termination reactions of the α-hydroxypropyl radical. The observation of stable yellow corrinoids by UV-visible spectroscopy (see above) and thin-layer chromatography on cellulose of the concentrated reaction mixture (yellow, light-stable spots $R_{B12}$ = 0,95 and 0,19-0,76 cf. $R_{B12}$ of $B_{12a}$ = 0,19 in sec-butanol/acetic acid/water 100:1:50) suggests that the major termination pathway of the α-hydroxypropyl radical is attack on the corrin ring.

The α-hydroxypropyl radical could react with the corrin ligand either by attaching to the corrin ring or by abstracting a hydrogen or methyl radical. X-ray crystallography of a stable yellow corrinoid[13] (formed by attack of ascorbic acid on dicyanocobrynic acid heptamethylester) showed attachment of a

hydroxy group at C-5 (Section 1.6). In the photolysis of methylcobalamin[96] and the flash photolysis of adenosylcobalamin[93] high concentrations of α-hydroxyprcpyl free radicals were pr.sumably generated within a short time. In the homolysis of neopentylcobalamin there is probably a low concentration of α-hydroxypropyl free radicals at any one time so that attack on the corrin ligand is preferred to dimerization or oxidation of the radical.

6.4.4 Reactions in the presence of thiols

Neopentylcobalamin in acid solution pH = 2,2, under nitrogen was stable (≤ two per cent decomposition) to cysteine (0,1M) for at least one hour. Neopentylcobalamin in nitrogen in the presence of cysteine (0,028M) at neutral pH was decomposed to give $B_{12r}$ at the rate of fourteen per cent in one hour and in the presence of thioglycollate (0,04M) at neutral pH was decomposed to give $B_{12r}$ with a half-life of twenty minutes (Table 6.1). The isosbestic points for these reactions were obscured by the presence of small amounts of thiolatocobalamin (absorbing at ~370nm) probably formed because of residual traces of oxygen in the reaction mixture. The amount of $B_{12r}$ observed was about sixty per cent of that expected from the complete decomposition of neopentylcobalamin by cysteine or thioglycollate. On admitting air to the reaction mixture the thiolatocobalamin corresponding to cysteine or thioglycollate is formed (shown by comparison with the corresponding thiolatocobalamin formed from $B_{12a}$ with cysteine or thioglycollate) together with some stable yellow corrinoid (as shown by a peak at 467nm). The decomposition of

neopentylcobalamin is therefore accelerated by thiols.

Gas chromatography of the gas phase of a reaction mixture containing neopentylcobalamin, thioglycollate and phosphate buffer at pH = 5,6 in nitrogen (Section 6.3.2) showed the presence of a peak which increased in intensity over three hours and which had the same retention time as neopentane. This peak was not present at the start of the reaction and was not observed in the corresponding blank reaction. A large number of indistinct peaks at higher retention times (possibly due to the decomposition of thioglycollic acid on the column) were observed for both the thioglycollate reaction mixture and for the blank. These peaks prevented the detection of any disulphide which might have been formed from the thiol radical. Another possible fate of the thiol radical is attack on the corrin ring to give stable yellow corrinoids and this probably happens, at least to some extent, in the neopentylcobalamin and thioglycollate reaction mixture.

As with the isopropanol reaction with neopentylcobalamin there is uncertainty whether the initial cobalamin product is $B_{12r}$. It is possible that the initial product is $B_{12s}$ or thiolatocobalamin. $B_{12r}$ is formed by the decomposition of $B_{12s}$ with the liberation of hydrogen in acid and neutral solution.[9, 107, 108] At pH's below seven and on the time-scale of these reactions the possibility that $B_{12s}$ is the primary product cannot be eliminated.

It is well known that thiols can act as reducing agents

(giving $B_{12r}$) as well as ligands for cobalamins. Thiols such as cysteine or thioglycollate can coordinate to $B_{12a}$ to give a thiolatocobalamin which is then reduced to $B_{12r}$ at a rate which increases with increasing pH.[5p] At pH's where the reaction of neopentylcobalamin with thiols proceeds rapidly ie. above 5,6, the rate of reduction of thiolatocobalamins is fairly fast eg. for thioglycollate at pH = 5,8 the half-life for the conversion of the thiolatocobalamin to $B_{12r}$ is fifty minutes. Thiolatocobalamins cannot be eliminated as primary products for the attack of thiols on neopentylcobalamin.

6.4.5 Reactions in the presence of imidazole (under nitrogen)

The reaction of neopentylcobalamin with imidazole (1,3M) gives a final spectrum with $\alpha$, $\beta$ and $\gamma$peaks at 538, 508 and 357nm respectively. This spectrum is identical to that obtained from the 'instantaneous' reaction of $B_{12a}$ with imidazole and can be assigned to imidazolecobalamin. The overall reaction, followed at 538nm, gives a half-time of fifteen to twenty minutes ie. imidazole accelerates the decomposition of neopentylcobalamin. The reaction appears to proceed in at least two stages: an intermediate is clearly seen which has a band at ~508nm but less absorption about 540nm and a less obvious shoulder at ~410nm. The intermediate has not been identified. The first stage gives isosbestic points at 374nm and 490nm and follows reasonable first-order kinetics with $t_{\frac{1}{2}}$ = five minutes.

The speeding up of the decomposition of neopentylcobalamin

by imidazole is probably due to attack of the neopentyl free radical on the imidazole ring, resulting in a shift in the equilibrium for homolytic fission in favour of homolysis. The reaction is probably analogous to the cyclization of the adenosyl radical on to the meso carbon of the imidazole ring of adenine during the anaerobic photolysis of adenosylcobalamin[99] and to the corresponding reaction observed for 2,3'-isopropylidene-5'-deoxyuridinyl cobalamin[101] (Section 6.2.1). The formation of a cobalt (III) complex rather than $B_{12r}$ is presumably due to the reduction of the radical formed by the attack of neopentyl radical on imidazole by $B_{12r}$ with the concomitant formation of a cobalt (III) complex.

Neopentylcobinamide also reacts with imidazole but much more slowly than neopentylcobalamin. The final spectrum (peaks at 538, 508 and 357nm) is ascribed to bis-imidazole-cobinamide and is very similar to the final spectrum of imidazolecobalamin. The reaction also goes via two stages and the overall reaction shows the formation of about thirty per cent product in one hour.

The decomposition of neopentylcobinamide by imidazole is probably due to the formation of a transient six-coordinate complex with imidazole coordinated in the 'lower' position of neopentylcobinamide. The decomposition is unlikely to proceed via the five-coordinate neopentylcobinamide as intermediate because this species is stable in the presence of reagents (eg. oxygen, isopropanol) which promote homolysis of the Co-C bond but which cannot coordinate to the cobalt.

By comparing the equilibrium constants for the coordination of benzimidazole (sixty per cent 'base-on')(Section 5.3.1) and imidazole (log K< -1,5)(Section 6.4.1) and the relative rates of reaction of neopentylcobalamin and neopentylcobinamide with imidazole it can be seen that the reaction of neopentylcobalamin must be due to the 'base-on' cobalamin and not to a 'base-off' form or to the complex where benzimidazole has been substituted by imidazole.

The effect of reagents which are known to promote homolytic fission of methylcobalamin and adenosylcobalamin (in light) (section 6.2) on the 'base-on' form of neopentylcobalamin can be summarized as follows:

(a) decomposition is accelerated by oxygen, thiols (cysteine and thioglycollate), alcohols (n-propanol and isopropanol) and imidazole;

(b) the order of reactivity (if the effect of reagents is assumed to be proportional to concentration) is oxygen >> thioglycollate > cysteine >> isopropanol > n-propanol > no added reagent;

(c) neopentane is formed in the presence of thioglycollate or isopropanol. It can therefore be concluded that neopentylcobalamin decomposes by the same mechanism, namely homolytic fission, as methylcobalamin and adenosylcobalamin.

## 6.5 Mechanism of decomposition of neopentylcobalamin

It was shown in the previous section, by analogy with the homolysis of methylcobalamin and adenosylcobalamin, that neopentylcobalamin decomposes by homolytic fission. However, homolysis of methylcobalamin or adenosylcobalamin requires light or elevated temperatures (Section 6.2) while homolysis of neopentylcobalamin occurs in the dark and at room temperature. Because the Co-C bond is long and weak (due to steric interaction of the methyl groups of the neopentyl ligand with the side-chains of the corrin ring) enough thermal energy is available at room temperature for homolytic fission of the Co-C bond of a small percentage of neopentylcobalamin molecules to occur, allowing the equilibrium

$$\text{Co-R} \rightleftharpoons \text{Co(II)} + \text{R}\cdot$$

to be set up. That the lability of neopentylcobalamin is due to the interaction of the neopentyl ligand with the side-chains of the corrin ligand is supported by the observation that neopentylcobaloxime[109] (where the ligand has no projecting side-chains) is stable. Neopentylcobalamin therefore provides a model for the homolytic fission of adenosylcobalamin during the isomerase enzyme reactions (Section 9.1)

The lability of the six-coordinate forms (viz. neopentylcobalamin with benzimidazole coordinated in the sixth position and neopentylcobinamide with imidazole coordinated in

the sixth position) is much greater than that of the five-coordinate forms (viz neopentylcobalamin in the protonated, 'base-off' form and neopentylcobinamide). This can be ascribed to relief of steric strain in the five-coordinate complexes due to movement of the cobalt atom out of the plane of the corrin ring (Section 4.5.1).

# CHAPTER 7 - B-ELIMINATION, HOMOLYTIC FISSION AND OTHER REACTIONS OF ORGANOCOBALAMINS

## 7.1 Introduction

Reactions which involve the decomposition (by breaking of the Co-C bond) of organocorrinoids with simple alkyl ligands having no functional groups will be discussed in this chapter. The two alternative mechanisms for spontaneous decomposition of alkylcobalamins viz. homolytic fission and $\beta$-elimination will be considered and it will be shown that the rate at which simple alkylcobalamins spontaneously decompose depends mainly on a single factor, ie. the amount of steric compression caused by the organoligand, whichever mechanism is operative. The cleavage of the Co-C bond of organocobalamins in the presence of oxidizing agents (cobalt boride in the presence of air, cerium (IV) sulphate and vanadyl sulphate in the presence of air) and reducing agents (sodium borohydride) is discussed.

## 7.2 $\beta$-elimination and homolytic fission

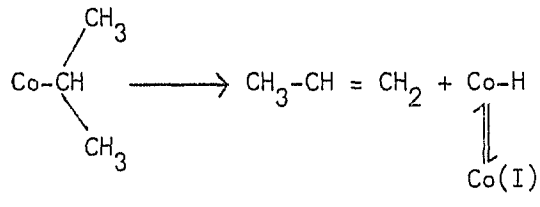
Two different mechanisms have been proposed for the decomposition of alkylcobalamins in the absence of added reagents. These are homolytic fission (discussed in Chapter 6) which is the mechanism of thermolysis for alkylcobalamins with no hydrogen in the $\beta$-position (eg. methylcobalamin, neopentylcobalamin) and

β-elimination[24,25,110] which is probably the mechanism of thermolysis for most other alkylcobalamins. Homolytic fission under anaerobic conditions in aqueous solution is reversible (Chapter 6), β-Elimination is potentially reversible but in aqueous solution the cobalt hydride intermediate is irreversibly decomposed to give $B_{12r}$.[24] β-Elimination occurs at the same rate in the presence and absence of oxygen[24] while homolytic fission is accelerated by oxygen (Chapter 6). Acceleration of decomposition of an alkylcobalamin by oxygen can be used as evidence that homolytic fission is occurring.



The fact that secondary alkylcobalamins, eg. isopropyl[21] and cyclohexylcobalamin,[22,23] are unstable even at room temperature and in the dark has been known for a long time. Isopropylcobalamin has been prepared by the reaction of propene with hydridocobalamin which is the protonated form of $B_{12s}$ and other organocobalamins have been prepared by the analogous reaction of alkenes with hydridocobalamin.[12]

Grate and Schrauzer[24] proposed that isopropylcobalamin and other secondary organocobalamins, including cycloalkylcobalamins, decompose spontaneously by β-elimination to give $B_{12s}$ and propene or an alkene according to the equation.



They showed that the rate of decomposition of isopropylcobalamin in aqueous solution is not affected by pH (above the pKa of the alkylcobalamin) or the presence or absence of oxygen (oxygen is not involved in the rate-determining step for the reaction), gives $B_{12s}$ (shown by the detection of methylcobalamin in ninety-five per cent yield on addition of methyl iodide to the reaction mixture and shows an isotope effect which must be interpreted as elimination of a β-hydrogen in the rate-determining step. They also found that the rates of decomposition of cycloalkylcobalamins (cycloctyl > cycloheptyl > cyclopentyl > cyclohexyl) showed the same order as the rates for the decomposition of cycloalkylamine oxides which is a known β-elimination reaction. This suggests that the decomposition of cycloalkylcobalamins also occurs by β-elimination but is not conclusive because other reactions[111] (eg. the free radical decomposition of azoalkanes R-N = N-R where

$$R = \left(CH_2\right)_{n-1}\overset{CN}{\overset{|}{C}}\!\!<\quad , \; n = 5\text{-}8)$$

show the same rate dependence.

7.2.1 Experimental procedure

All the reactions were followed by UV-visible spectroscopy as described in section 6.3.1 for neopentylcobalamin. The rates of reactions were measured at pH = 1 (0,05M sulphuric acid) or neutral pH (unbuffered deionized water or phosphate buffer pH = 6,4-6,9) for cobalamin solutions of approximately $3 \times 10^{-5}$M. Stable alkylcobalamin solutions were prepared by dissolving solid alkylcobalamin in 0,05M sulphuric acid or deionized water. Less

stable alkylcobalamins (cyclopentyl, cyclohexyl and isopropyl) were prepared *in situ* in the spectrophotometer cell as described in section 3.4 The rates of decomposition of alkylcobalamins under nitrogen and in the presence of air are given in Table 7.1

7.2.2 Reactions under nitrogen

All the alkylcobalamins studied are stable ( $\leq$ two per cent decomposition in one hour) at pH = 1,0-1,2 at 25°C. At higher temperatures (50 and 80°C) methyl- ethyl- and cyclopropylcobalamin still show no decomposition and cyclobutylcobalamin shows only a small amount (seven per cent in one hour) of decomposition at 80°C. Cyclohexylcobalamin at 50°C shows very little decomposition ($\leq$five per cent in one hour) to give $B_{12r}$ but for isopropyl- and cyclopentylcobalamin the reaction is faster, producing twenty per cent and thirty-six per cent $B_{12r}$ in one hour, respectively. At 80°C decomposition is more rapid ($t_{\frac{1}{2}}$ = twenty-five minutes, five minutes and seven minutes for cyclohexyl-, cyclopentyl- and isopropylcobalamin respectively) and the rate of decomposition is about five times faster for cyclopentyl- and isopropylcobalamin than for cyclohexylcobalamin. Complete conversion to $B_{12r}$ was observed for these reactions.

At pH = 6-7 methyl-, ethyl-, cyclopropyl, cyclobutyl- and isobutyl-cobalamin show no reaction at 25°C. Isobutylcobalamin, at 50°C, decomposes to give $B_{12r}$ (twenty-one per cent in one hour); methyl-, ethyl-, cyclopropyl- and cyclobutylcobalamin were not checked at 50°C. Cyclopentyl-, cyclohexyl- and isopropylcobalamin

Table 7.1 - Rates of Decomposition of Organocobalamins

| pH | Gas phase | Temperature (°C) | Methyl | Cyclopropyl | Ethyl | Cyclobutyl | Isobutyl | Cyclohexyl | Isopropyl | Cyclopentyl |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | $N_2$ | 25 | - | - | - | - | | - | - | - |
| | | 50 | - | - | - | - | | ≦ 5% | 20% | 36% |
| | | 80 | - | - | - | 7% | | 25 minutes | 7 minutes | 5 minutes |
| 1 | air | 25 | - | - | - | - | 8% | | | |
| | | 50 | - | - | - | 3% | | | | |
| | | 80 | ≦ 5% | ≦ 5% | 10% | 23% | | | | |
| 6-7 | $N_2$ | 25 | - | - | - | - | - | 1 hour[b] | 2 minutes | 1 minute |
| | | 50 | | | | | 21% | | | |
| 6-7 | air | 25 | - | - | - | - | - | 21 minutes[c] | [d] | 1 minute |
| | | 50 | - | - | - | - | 21% | | | |
| | | 80 | - | - | 30 minutes | 10% | | | | |

[a] All solutions contained approximately $3 \times 10^{-5}$M cobalamin in 0,05M $H_2SO_4$ or, for neutral solution in either phosphate buffer pH 6,4-6,9 (cyclopentyl, cyclohexyl, isopropyl, neopentyl) or unbuffered deionised water pH 6 (all others). Where decomposition is observed, the rate is given as either (i) the half-time of reaction or (ii) the percent decomposed in one hour; '——' denoted no detectable (≦ 2%) decomposition in one hour.

[b] 50% in 1 hour and 49% in 1 hour in two separate experiments.

[c] $t_{\frac{1}{2}}$ = 20 and 22 minutes in two separate experiments.

[d] Grate and Schrauzer[24] have shown that the presence of air has no effect on the rate of decomposition of isopropyl-cobalamin at pH 7.

decompose at 25°C to give $B_{12r}$; cyclohexylcobalamin rather slowly ($t_{\frac{1}{2}} \sim$ one hour) and cyclopentyl and isopropylcobalamin rapidly ($t_{\frac{1}{2}} <$ one and $t_{\frac{1}{2}} <$ two minutes) respectively. It can be seen that isopropyl-, cyclopentyl- and cyclohexylcobalamin decompose more rapidly at neutral pH than at acid pH. The rates of decomposition of organocobalamins under nitrogen are given in Table 7.1.

The formation of $B_{12r}$ rather than the $B_{12s}$ expected for β-elimination, at neutral and acid pH (no peak is seen around 386nm which is the main band for $B_{12s}$) can be ascribed to the known rapid decomposition of $B_{12r}$ to $B_{12s}$ with liberation of hydrogen in acid and neutral solutions and the acceleration of this decomposition by buffer anions.[9,107,108] $B_{12s}$ was observed during the decomposition of cyclopentylcobalamin and cyclohexylcobalamin at pH = 12 and the rate of decomposition was slowed down due to a recycling reaction of $B_{12s}$ with excess cyclopentyl or cyclohexyl bromide present in the spectrophotometer cell (section 3.4). Such a recycling reaction is unlikely to occur to any significant extent at pH's $\leq 7$ because conversion of $B_{12r}$ to $B_{12s}$ is rapid[9,107,108] and reaction of $B_{12s}$ with secondary alkyl bromides is slow (section 3.4).

7.2.3 Reactions in air and in isopropand

The rate of decomposition by β-elimination should not be affected by the presence of oxygen or of isopropanol but homolytic fission should be accelerated by oxygen or isopropanol (Chapter 6). In order to distinguish between homolytic fission and β-elimination

the reactions of alkylcobalamins were studied in air and in isopropanol.

Methyl-, ethyl-, cyclpropyl- and cyclobutylcobalamin are all stable (≤two per cent decomposition in one hour) in the presence of air at pH = 1 and 25°C but decompose slowly at 80°C (Table 7.1) giving $B_{12a}$. The faster decomposition at 80°C in the presence than in the absence of air suggests that, for these alkylcobalamins, some homolytic fission (in addition to β-elimination) occurs at higher temperatures. Isobutylcobalamin shows slow decomposition (eight per cent in one hour) in air giving $B_{12a}$ at pH = 1 and 25°C ie. it decomposes more rapidly than neopentylcobalamin (section 6.4.2) under the same conditions. Isobutylcobalamin presumably decomposes by β-elimination and neopentylcobalamin by homolytic fission (chapter 6). Isopropyl-, cyclopentyl- and cyclohexylcobalamin are stable at pH = 1 and 25°C in the presence of air except when traces of cobalt boride are present (section 7.4.1).

Methyl-, ethyl-, cyclopropyl- and cyclobutylcobalamin are stable at 25°C or 50°C at neutral pH in the presence of air but ethyl- and cyclobutylcobalamin show some decomposition at 80°C (Table 7.1). Isobutylcobalamin at 50°C decomposes at the same rate (twenty-one per cent in one hour) as in the absence of cir. This means that β-elimination is the sole decomposition pathway and no homolytic fission occurs for isobutylcobalamin. Isopropylcobalamin and cyclopentylcobalamin both decompose very

rapidly even under nitrogen and the rate of decomposition in air appears to be similar to that in nitrogen. For cyclohexylcobalamin the rate of decomposition in the presence of air is greater than that in the presence of nitrogen suggesting that some of the cyclohexylcobalamin decomposes via a homolytic pathway in the presence of air. The rates of decomposition of alkylcobalamins in in air are given in Table 7.1. In all cases the product of decomposition was $B_{12a}$.

Cyclopentylcobalamin and cyclohexylcobalamin are stable ($\leq$ two per cent decomposition in one hour) in the presence of 1,0M isopropanol under nitrogen. For cyclohexylcobalamin the rate of decomposition at neutral pH is slightly accelerated ($t_{\frac{1}{2}}$ = forty minutes) in the presence of 1,0M isopropanol under nitrogen, giving $B_{12r}$. This indicates that cyclohexylcobalamin decomposes partially by homolytic fission.

7.2.4 Homolytic fission and β-elimination of cyclohexylcobalamin

As shown in the previous section cyclohexylcobalamin decomposes partially by β-elimination (some decomposition is observed under nitrogen) and partially by homolytic fission (decomposition is accelerated by air or isopropanol). The observation of cyclohexane as well as cyclohexene in the gas phase after decomposition of cyclohexylcobalamin in the presence of dithioerythrol[22] also suggests that homolytic fission can occur in parallel with β-elimination; cyclohexane presumably arises from abstraction of a hydrogen atom from dithioerythrol and

cyclohexene from β-elimination. Homolytic fission is observed for cyclohexylcobalamin, where β-elimination is slow, but not for cyclopentyl- or isopropylcobalamin where β-elimination is fast.

The enhanced reaction rates observed in the presence of air at 80°C and pH = 1 for ethyl-, cyclopropyl- and cyclobutyl cyclobutylcobalamin suggest that homolytic fission could be a mechanism of decomposition for these alkylcobalamins, at least at higher temperatures.

The decomposition of cyclohexylcobalamin partially by homolytic fission is evidence that a secondary alkyl radical can react with $B_{12r}$ to give an alkylcobalamin. Another example of reaction of a secondary alkyl radical with $B_{12r}$ is the reaction of cyclodecyl iodide with $B_{12s}$ via electron transfer from $B_{12s}$ to cyclodecyl iodide giving cyclodecyl radical and $B_{12r}$ which then react together.[46]

## 7.3 Correlation of the rates of decomposition of alkylcobalamins with steric factors

Even allowing for decomposition of alkylcobalamins by two different mechanisms (homolytic fission and β-elimination) there is a clear trend in the decomposition rates of alkylcobalamins within the different series (section 4.1.2). The rate of decomposition increases as follows (mechanism of decomposition given in brackets):- methyl (homolytic fission) < ethyl (β-elimination) < isopropyl (β-elimination); ethyl (β-elimination) < isobutyl (β-elimination) < neopentylcobalamin (homolytic fission); cyclopropyl (β-elimination) < cyclobutyl (β-elimination) <

cyclohexyl (homolytic fission and β-elimination) < cyclopentyl (β- elimination). The order for the rates of decomposition is, apart from two exceptions, exactly the same as that obtained from the spectra and equilibria of alkylcobalamins (sections 4.2 and 4.3). In acid solution and in the presence of air the decomposition of isobutylcobalamin is faster than that of neopentylcobalamin. The reason for this is probably that β-elimination, when available, is the more facile pathway for decomposition of alkylcobalamins. The order for the rate of decomposition of cyclopentylcobalamin and cyclohexylcobalamin is inverted compared to the order of pKa's and spectra. Cyclopentylcobalamin decomposes entirely by β-elimination and cyclohexylcobalamin partially by β-elimination and partially by homolytic fission. For both mechanisms of decomposition the transition state will have a large degree of $sp^2$ character[111] (partial formation of an alkene for β-elimination and partial formation of a planar free radical for homolytic fission). The formation of a $sp^2$ centre leads to a decrease in comformational strain in the case of a five-membered ring and to an increase in the case of a six-membered ring.[111] Thus steric factors in the transition state are also important in determining the rates of decomposition of alkylcobalamins.

Considering all the alkylcobalamins studied, the overall order for the increase in rate of cleavage of the Co-C bond is methyl ≤ cyclopropyl < ethyl ~ cyclobutyl < isobutyl < neopentyl ~

cyclohexyl < cyclopentyl ~ isopropyl which is very similar to that obtained from the spectra and equilibria of alkylcobalamins (sections 4.2 and 4.3) except for the change in order of cyclobutyl and isobutylcobalamin. This is presumably also due to steric factors in the transition state rather than in the initial complex with the β-hydrogen of isobutylcobalamin more favourably placed for β-elimination than the β-hydrogen of cyclobutylcobalamin.

It can be seen that alkylcobalamins, where the organoligand causes steric compression, are more stable in the five-coordinate forms (in acid solution) than in the six-cordinate forms, in line with the expected decrease in lability with relief of steric strain in the five-coordinate forms. This effect is observed not only when the lower axial ligand is benzimidazole but also for less bulky groups such as imidazole, ammonia and cyanide[22,23] (section 6.4.5).

For both homolytic fission and β-elimination the lability of the Co-C bond can be ascribed to distortion of the bond angles around $C_\alpha$ and the consequent lengthening and weakening of the Co-C bond. Subtracting out the rate of β-elimination for cyclohexylcobalamin gives approximately the same rate of homolytic fission for cyclohexylcobalamin as for neopentylcobalamin. This means that the Co-C bond can be labilized towards homolytic fission to about the same extent by increasing or decreasing the $Co\text{-}\hat{C}_\alpha\text{-}C_\beta$ bond angle.

## 7.4 Cleavage of the Co-C bond using oxidizing and reducing agents

### 7.4.1 Decomposition of cyclopentyl- and cyclohexylcobalamin by cobalt boride

For cyclopentylcobalamin and cyclohexylcobalamin an unexpected decomposition reaction, giving $B_{12a}$, occurred at pH = 1 when air or oxygen was passed through the solution. The reaction proceeded very rapidly during the first minute and then stopped or slowed down greatly before complete formation of $B_{12a}$ occurred. This reaction was traced to cobalt boride in the presence of oxygen. During the preparation of cyclopentyl- and cyclohexylcobalamin, cobaltous nitrate ($1,2 \times 10^{-5}$M) was present in solution in order to catalyse the reduction of $B_{12a}$ to $B_{12s}$ by sodium borohydride[9] (section 3.2). Cobalt boride ($Co_2B$) can be formed by the sodium borohydride reduction of cobalt (II) salts in aqueous solution.[49] Occasionally a small amount of a solid black compound (presumably cobalt boride) was seen during reduction by borohydride.

It was shown that cyclohexylcobalamin prepared in the absence of added cobalt nitrate was stable when air was passed through the reaction mixture. If some freshly prepared cobalt boride was added to the reaction mixture decomposition to $B_{12a}$ occurred when air was passed through the solution. This shows that catalysis by cobalt boride, rather than inhibition by some other reagent, is taking place. Cobalt boride was itself inactivated in the presence of air since a solution of cobalt

boride which had stood in air for one hour failed to restart the decomposition of cyclohexylcobalamin to $B_{12a}$. Neither cobalt (II) chloride nor copper (II) sulphate was capable of restarting the decomposition to $B_{12a}$. No comparable reaction was observed at pH = 1 under nitrogen.

It therefore appears that both cobalt boride and oxygen are required for decomposition of cyclohexylcobalamin and cyclopentylcobalamin to $B_{12a}$ at pH = 1. Transition metal borides such as cobalt boride are known to catalyse a number of reactions eg. decomposition of hydrogen peroxide and decomposition of hydrazine to ammonia.[112]

It has been observed previously that organocobalamins are decomposed in the presence of sodium borohydride and added copper (II) salts.[9] Probably this decomposition is catalysed by copper boride.

The observation of the decomposition of cyclopentyl- and cyclohexylcobalamin by cobalt boride under oxidizing conditions suggested that it might be interesting to observe the effect of other oxidizing agents on sterically strained organocobalamins. The effects of ceric sulphate and vanadyl sulphate on cyclohexylcobalamin were therefore determined.

7.4.2 Reaction of cyclohexylcobalamin with cerium (IV) sulphate

Cyclohexylcobalamin reacts with cerium (IV) sulphate in dilute sulphuric acid to give $B_{12r}$. The conditions of reaction were:- concentration of cyclohexylcobalamin = $3 \times 10^{-5}$M,

concentration of sulphuric acid = 0,05M, pH = 1,2. Under these conditions $B_{12r}$ (observed spectroscopically) is produced very rapidly (the reaction was complete in one minute). Addition of a further aliquot of cerium (IV) sulphate (to give a final concentration of $5,0 \times 10^{-4}$M) gave rise to the formation of $B_{12a}$. $B_{12r}$ ($3 \times 10^{-5}$M) in the presence of cerium (IV) sulphate ($2,5 \times 10^{-4}$M) and sulphuric acid (0,05M) was immediately oxidized to $B_{12a}$. No attempt was made to isolate the organic products of the reaction. Methylcobalamin under the same conditions showed no reaction over thirty minutes ie. less than five per cent $B_{12r}$ was formed in thirty minutes.

Organocobaloximes can be cleaved by the action of one-electron oxidants such as cerium (IV) or iridium (IV) or by electrochemical oxidation to give cobaloxime (II).[113-115] It has been proposed that the organocobaloxime is oxidized to give a radical cation complex (which is formally Co(IV) as shown by ESR[116]) and that the cleavage of the Co-C bond results from subsequent nucleophilic attack (possibly by solvent) at $C_\alpha$.[113,115]

It appears likely that the decomposition of cyclohexylcobalamin by cerium (IV) proceeds by a mechanism similar to that proposed for the oxidative cleavage of the Co-C bond of organocobaloximes. This would involve oxidation of cyclohexylcobalamin by Ce(IV) to give a formally Co(IV) complex followed by rapid nucleophilic attack by $H_2O$ on the radical-cation, giving $B_{12r}$ and an alcohol corresponding to the radical. The

observation of $B_{12r}$ as product is consistent with this mechanism but obviously more work is needed for confirmation.

Other workers[117] have shown that methylcobalamin is decomposed by iridium (IV) giving $B_{12a}$.

7.4.3 Reaction of cyclohexylcobalamin with vanadyl sulphate

Cyclohexylcobalamin ($3 \times 10^{-5}$M) reacts with vanadyl sulphate ($2,4 \times 10^{-4}$M) at pH = 1,0 in the presence of air to give $B_{12a}$ with a half-life of less than one minute.

7.4.4 Decomposition of alkylcobalamins by sodium borohydride

Organocobalamins in general are stable in the presence of sodium borohydride[9] but organocobalamins such as ethinyl, trifluorethyl and sulphomethyl[118] and unspecified sterically hindered alkylcobalamins[119] are decomposed by borohydride. Neopentylcobalamin ($3 \times 10^{-5}$M) is rapidly cleaved ($t_{\frac{1}{2}} \leq 2$ minutes) by 0,5M sodium borohydride (section 3.5). It appears that the long, weak bond of sterically hindered organocobalamins is susceptible to reductive cleavage by sodium borohydride.

7.5 Summary

The spontaneous decomposition of simple alkylcobalamins via homolytic fission and β-elimination is discussed and it is shown that decomposition by the two mechanisms can occur in parallel as in the case of cyclohexylcobalamin. It is shown that the rate of spontaneous decomposition of alkylcobalamins (irrespective of the mechanism of decomposition) increases with increasing steric compression caused by the organoligand. This is ascribed to the

lengthening and weakening of the Co-C bond caused by increased steric compression. For organocorrinoids with sterically bulky organoligands five-coordinate species are more stable than the corresponding six-coordinate forms.

It is shown that decomposition of cyclohexylcobalamin via oxidative cleavage (using cerium (IV), vanadium (IV) or cobalt boride) occurs even in the more stable five-coordinate form and that neopentylcobalamin undergoes rapid reductive cleavage by sodium borohydride.

# CHAPTER 8 - ISOMERIZATION OF CYCLOPROPYLMETHYLCOBALAMIN TO 3-BUTENYLCOBALAMIN

## 8.1 Introduction

Among the enzyme reactions catalysed by adenosylcobalamin are the "carbon-skeleton" rearrangements viz the interconversion of L-glutamate and L-threo-$\beta$-methylaspartate, methylmalonyl-coenzyme A and succinyl-coenzyme A, $\alpha$-methyleneglutarate and methylitaconate.[2,7,14-16] The enzymes and the reactions they catalyse are given in Table 1.1. A key step in the mechanism of these enzymes is the rearrangement of the substrate, after abstraction of a hydrogen atom, to give the product (section 1.7 and Fig.1.5). The substrate could possibly rearrange as a free radical, a carbonium ion or carbanion or while attached via a Co-C $\sigma$ bond to the cobalamin. Cobalamins with organic ligands having the same carbon skeleton as the substrates for reactions 2 and 3 in Table 1.1 have recently been prepared by other workers;[20,26-34] these will be discussed in Chapter 9.

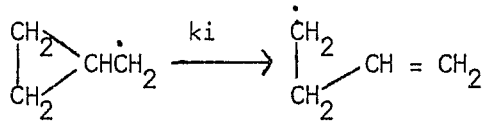
In order to provide further evidence on the machanism of the "carbon-skeleton" rearrangements, in particular whether or not rearrangement is likely to occur with the ligand coordinated to cobalt, the possible interconversions of cyclopropylmethyl, 3-butenyl and cyclobutylcobalamin were studied. These particular organocobalamins were chosen for two reasons.

(1) Interconversions of cyclopropylmethyl, 3-butenyl and cyclobutyl carbonium ions and of cyclopropylmethyl and 3-butenyl radicals have been intensively studied[120-122] (see section 8.2).

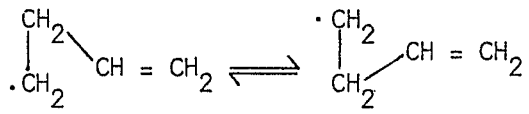
(2) There are no heteroatoms in the ligands to complicate the preparation and reactions of these organocobalamins and the interpretation of the results should therefore be easier.

## 8.2 Interconversion of $C_4H_7$ radicals and carbonium ions

The rearrangement of the cyclopropylmethyl radical to give the 3-butenyl radical is well known and has been extensively studied.[120,122] It rearranges rapidly to the 3-butenyl radical at temperatures as low as -120°C[123] with a rate constant of $k_i = 1,3 \times 10^8\ s^{-1}$ at 25°C.[124]




This rearrangement is apparently irreversible,[123] although the observed interconversion of 3-butenyl radicals




probably proceeds via a cyclopropylmethyl radical.[122,125] The cyclobutyl radical does not appear to be in ready equilibrium with the cyclopropylmethyl and 3-butenyl radicals.[120,122,126]

For reactions involving $C_4H_7^+$ carbonium ions the products are almost invariably a mixture of cyclobutyl, cyclopropylmethyl

and 3-butenyl species.[121,122] There is controversy over whether rearrangement occurs via discrete cyclobutyl, cyclopropylmethyl and 3-butenyl carbonium ions or via one or more non-classical carbonium ions.[127]

Cyclobutyl halide undergoes rearrangement to the 3-butenyl derivative on attempted formation of the cyclobutyl Grignard reagent and cyclopropylmethylmagnesium bromide rearranges to give 3-butenylmagnesium bromide. It appears, however, that these reactions do not proceed via a true carbanion.[128]

It therefore appears that for free radicals the only conversion observed is cyclopropylmethyl to 3-butenyl while for carbonium ions all $C_4H_7^+$ ions are interconvertible.

8.3 Experimental procedure and results

Cyclobutylcobalamin, cyclopropylmethylcobalamin and 3-butenylcobalamin as well as the corresponding cobinomides were prepared as described in Chapter 3 (sections 3.3 and 3.6).

The techniques used to study the reactions of these compounds were UV-visible spectroscopy in conjunction with column chromatography on CM cellulose and thin-layer chromatography in cellulose. The UV-visible spectra of cyclobutylcobalamin, cyclopropylmethylcobalamin and 3-butenylcobalamin are almost indistinguishable in neutral solution but there are slight differences in acidic solution. The differences between the spectra are too small to be used in monitoring the interconversion of these alkylcobalamins. In some cases the product was separated

from the reactant by column chromatography and the identity of the product verified by UV-visible spectroscopy.

The main technique used to study the reactions of cyclopropylmethylcobalamin and 3-butenylcobalamin was thin-layer chromatography in cellulose, using the solvent systems given in section 2.2.3. Cyclopropylmethylcobalamin and 3-butenylcobalamin were well separated in all three solvent systems used (see $R_{B12}$ values in Table 3.1) and gave clear, distinct spots. The spots were yellow-orange in colour when using solvents I or III (neutral or basic) and yellow when using solvent II (acidic) but turned red on exposure to sunlight due to conversion of the organocobalamins to $B_{12a}$. Reactions were followed by removing aliquots from the reaction mixture, freezing them in liquid nitrogen, and at the end of the experiment spotting the samples on a TLC plate. Half-lives for a reaction were determined by visually comparing the intensity of spots on thin-layer chromatography.

### 8.3.1 Isomerization of cyclopropylmethylcobalamin to 3-butenylcobalamin

Cyclopropylmethylcobalamin as a solid in the dark at room temperature slowly converted to 3-butenylcobalamin at a rate of approximately ten per cent per week as shown by thin-layer chromatography. When thin-layer chromatography showed the presence of cyclopropylmethylcobalamin and 3-butenylcobalamin in 1:1 proportions the sample was dissolved in water and chromatographed

on a CM cellulose column. Two organocobalamin fractions were obtained. The first fraction had a UV-visible spectrum identical to that of 3-butenylcobalamin at pH = 1 ( $\lambda$ = 455nm for the $\alpha\beta$ band) and at neutral pH ( $\lambda$ = 510nm for the $\alpha\beta$ band) and the second fraction had a UV-visible spectrum identical to that of cyclopropylmethylcobalamin at pH = 1 ( $\lambda$ = 442nm for the $\alpha\beta$ band) and at neutral pH ( $\lambda$ = 510nm for the $\alpha\beta$ band). The UV-visible spectra of cyclopropylmethylcobalamin and 3-butenylcobalamin in acidic and neutral solution are given in Figs. 8.1 and 8.2 respectively.

Thin-layer chromatography was used to study the rate of the isomerization of cyclopropylmethylcobalamin to 3-butenylcobalamin under various conditions (Table 8.1).

The reaction was studied in the dark at 60°C in air in the solid state and in aqueous solution at different concentrations. Fig.8.3 shows the time-course of a typical reaction. The range of concentrations over which the isomerization can be studied is rather narrow; it is limited by the solubility of cyclopropylmethylcobalamin in water and by the necessity to have a concentrated solution in order to detect the spots on the TLC plate. It can be seen that the effect of concentration on the isomerization of cyclopropylmethylcobalamin to 3-butenylcobalamin is small (Table 8.1).

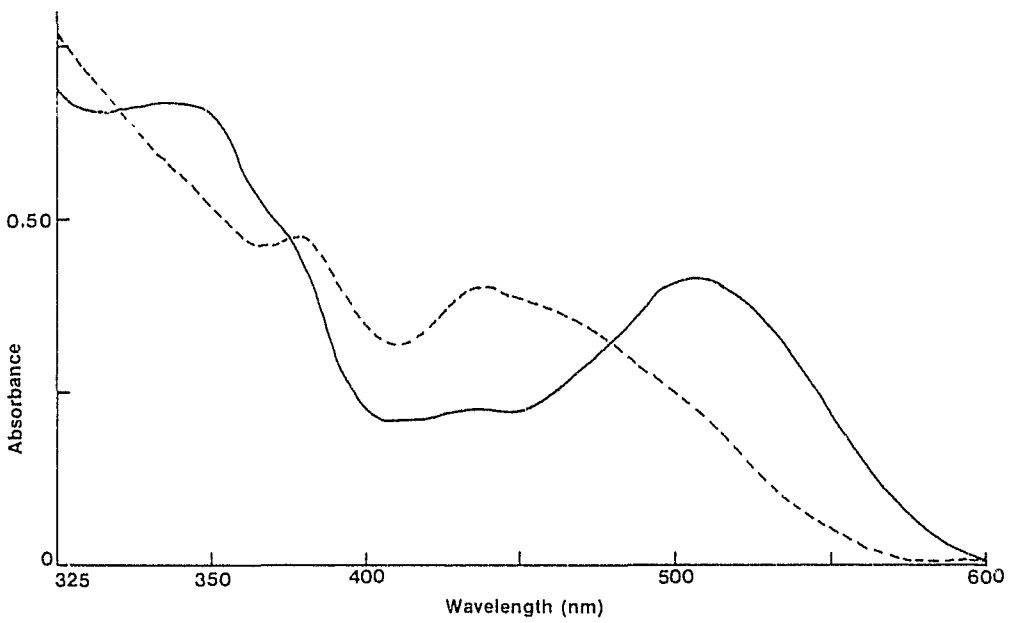



Fig.8.1 Spectra of $5,0 \times 10^{-5}$M cyclopropylmethylcobalamin at pH = 6,5 (——) and pH = 1 (---).

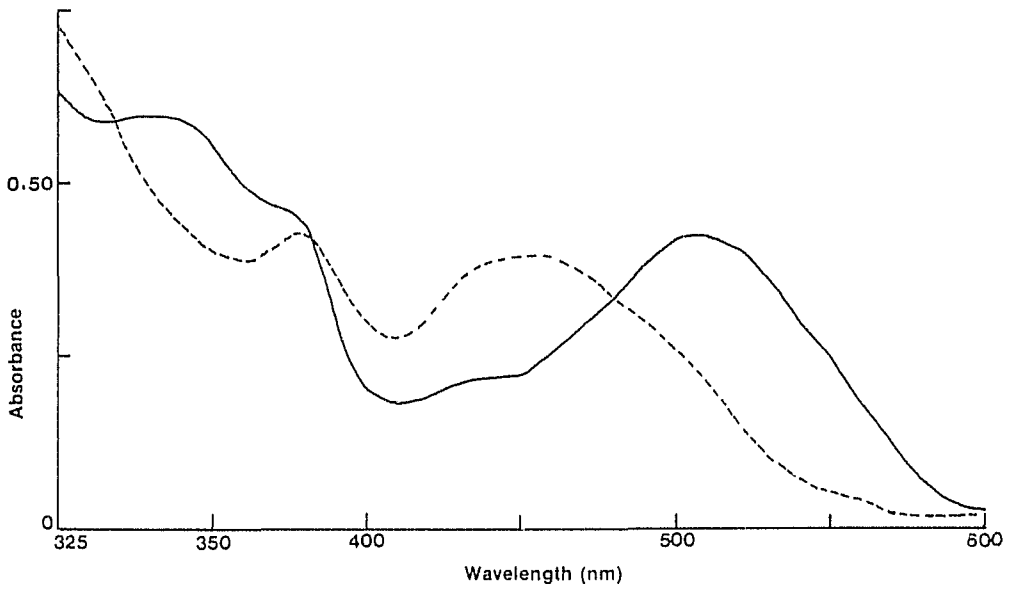



Fig.8.2 Spectra of $5,0 \times 10^{-5}$M 3-butenylcobalamin at pH = 6,5 (—— ) and pH = 1 (---).

Table 8.1 - Rates of Conversion of Cyclopropylmethylcobalamin to 3-Butenylcobalamin

| Solvent | Temperature (°C) | Gas phase | Dark or light | Concentration (mg/ml) | Rate of reaction[a] |
|---|---|---|---|---|---|
| solid state | 25°C | air | dark | | ~1 month |
| solid state | 60°C | air | dark | | ~6 hours |
| water | 60°C | air | dark | 20 | 165 minutes[b] |
| water | 60°C | air | dark | 7,6 | 120 minutes |
| water | 60°C | air | dark | 4,8 | 90 minutes |
| DMF | 60°C | air | dark | 20 | 180 minutes |
| Methanol | 60°C | air | dark | 20 | 150 minutes |
| Acetic acid | 60°C | air | dark | 20 | 120 minutes |
| water | 60°C | nitrogen | dark | 20 | 120 minutes |
| water | 25°C | air | light[c] | 20 | 5 minutes |

a The rate is given as the half-time of the reaction.

b Incorrectly quoted as 105 minutes in ref. 129.

c. Conditions: 200W tungsten lamp at a distance of 21cm.

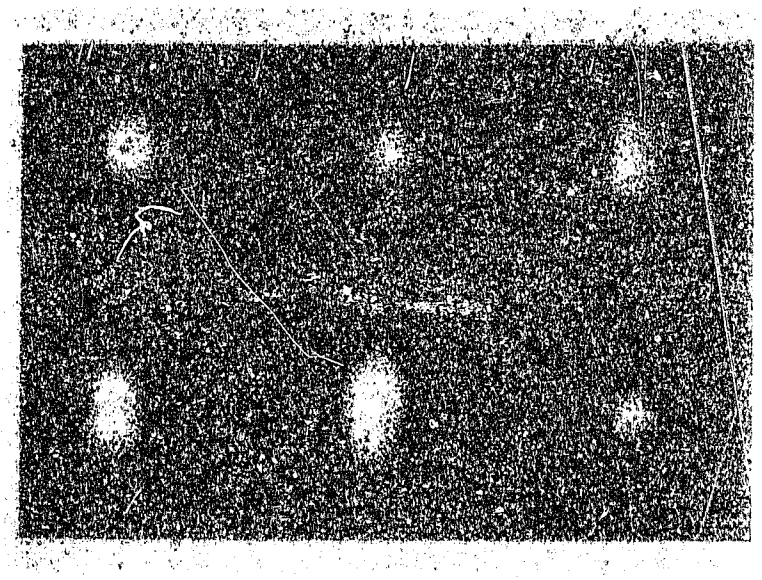



Fig.8.3 TLC of the conversion of cyclopropylmethylcobalamin to 3-butenylcobalamin in aqueous solution (20 mg/ml) at 60°C in air. Spots are shown for (1) 0 minutes, (2) 45 minutes, (3) 105 minutes, (4) 165 minutes, (5) 240 minutes, (6) 3-butenylcobalamin.

In order to determine the effect of solvent the rate of isomerization was measured in three different organis solvents as well as water. The dielectric contents of the solvents[130-132] used were; water (78,39), dimethylformamide (36,71), methanol (32,70) glacial-acetic acid (6,195). The reaction was also done using formamide (109,5) as the solvent but this interfered with the TLC, giving crescent shaped spots. The effect of solvent on the isomerization of cyclopropylmethylcobalamin to 3-butenylcobalamin is slight and does not vary systematically with the dielectric constant (Table 8.1).

UV-visible spectroscopy shows that cyclopropylmethylcobalamin is in the base-on form in water (Fig.8.1) but mainly or entirely in the protonated base-off form ($\lambda$ =442nm for the $\alpha\beta$ band) in acetic acid. The rate of isomerization of cyclopropylmethylcobalamin is not significantly affected by protonation and removal from coordination of the benzimidazole base (compare the rates given for acetic acid and for water in Table 8.1).

The presence of oxygen has no significant effect on the rate of isomerization of cyclopropylmethylcobalamin to 3-butenylcobalamin as shown by comparable experiments in air and in nitrogen (Table 8.1).

The rate of conversion of cyclopropylmethylcobalamin to 3-butenylcobalamin was dramatically enhanced in the presence of light in air (Table 8.1).

Besides 3-butenylcobalamin, the only products observed during the reaction of cyclopropylmethylcobalamin were a small amount of $B_{12a}$ and (occasionally) a decomposition product of 3-butenylcobalamin (section 8.3.3). The amount of $B_{12a}$ observed ($\leq$ ten per cent as estimated from TLC) was approximately the same for the thermal and photochemical reactions over the time-scale of the experiments (four hours and two hours respectively).

8.3.2 Isomerization of cyclopropylmethylcobinamide to 3-butenylcobinamide

Cyclopropylmethylcobinamide was mainly or entirely converted to 3-butenylcobinamide during preparation and purification as shown by UV-visible spectroscopy. The spectrum of 3-butenylcobinamide was very similar ($\lambda$ =455nm for the $\alpha\beta$ band) to that of the acidic form of 3-butenylcobalamin. The spectrum of cylclopropylmethylcobinamide after preparation and purification (which took three days) was the sume as that of 3-butenylcobinamide ($\lambda$ = 455nm for the $\alpha\beta$ band). This implies that all or most of the cyclopropylmethylcobinamide is converted to 3-butenylcobinamide during preparation and purification; cyclopropylmethyl bromide contained only 1,5 per cent 3-butenyl bromide (section 2.1.2) and the rates of reaction of the two bromides with $B_{12s}$ are similar (from observation of the colour changes during preparation). The rate of conversion of cyclopropylmethylcobinamide to 3-butenylcobinamide must be greater than or comparable to the

rate of interconversion of the corresponding cobalamins (ninety to ninety-five conversion during preparation, see section 3.3). Because cyclopropylmethylcobinamide and 3-butenylcobinamide underwent hydrolysis of some of the acetamide and propionamide side-chains, giving rise to additional compounds with low $R_{B12}$ values on TLC (section 3.6) the reactions of these compounds were not studied further.

8.3.3 3-Butenylcobalamin

3-Butenylcobalamin, as a solid at room temperature or in aqueous solution (20 mg/ml) at 60°C in the dark slowly decomposed to give $B_{12a}$ and sometimes, an unidentified organocobalamin which had a typical organocobalamin spectrum, showed a pH-dependent equilibrium and was light-sensitive (section 3.1.3). The organocobalamin had a low $R_{B12}$ value ($R_{B12}$ = 1,23 in solvent I (section 2.2.3) cf. cyclopropylmethylcobalamin $R_{B12}$ = 1,54 and 3-butenylcobalamin $R_{B12}$ = 1,59). The isomerization of 3-butenylcobalamin to cyclopropylmethylcobalamin was not observed.

8.3.4 Cyclobutylcobalamin

Cyclobutylcobalamin kept at room temperature in the solid state in the dark for three years showed no significant ($\leq$ two per cent) decomposition to $B_{12a}$ and no observable conversion to other organocobalamins.

Of the possible interconversions between cyclobutyl and cyclopropylmethyl and 3-butenyl the only one observed for organocorrinoids is cyclopropylmethyl to 3-butenyl. This

isomerization occurs for cobalamins in the 'base-on' and protonated 'base-off' (in acetic acid) forms and for cobinamides. The reaction is very slow at room temperature and is accelerated by light or heating to 60. The rate is relatively insensitive to oxygen, the dielectric constant of the solvent and concentration.

## 8.4 Discussion

### 8.4.1 Mechanism of isomerization of cyclopropylmethylcobalamin to 3-butenylcobalamin

The rearrangement of cyclopropylmethyl to 3-butenyl could occur either while the cyclopropylmethyl group is attached to cobalt or after the cleavage of the bond between the cobalt atom and the cyclopropylmethyl group. The fact that the reaction is accelerated at 60°C and considerably accelerated by light strongly suggests that the Co-C bond is broken before isomerization can occur. Attachment of the cyclopropylmethyl group to cobalt actually inhibits the rearrangement of cyclopropylmethyl to 3-butenyl since the cyclopropylmethyl free radical rearranges rapidly even at -120°C.[123]

The Co-C bond could be cleaved homolytically to give $B_{12r}$ and a free radical or heterolytically to give $B_{12s}$ and a carbonium ion. Because the only rearrangement observed is that of cyclopropylmethyl to 3-butenyl (which is apparently irreversible) and because there does not appear to be an equilibrium between cyclopropylmethyl, cyclobutyl and 3-butenylcobalamin the free

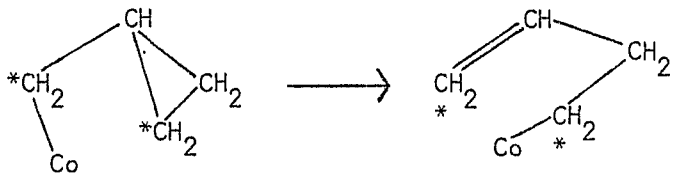
radical pathway appears to be more likely than the carbonium ion pathway. For free radicals only the conversion of cyclopropylmethyl to 3-butenyl is observed[120,122] but for carbonium ion reactions all $C_4H_7^+$ ions are in equilibrium[121] (section 8.2). Acceleration of the reaction by light is also suggestive of a free radical mechanism.

The rearrangement reaction could occur by a unimolecular mechanism or by a bimolecular displacement mechanism[133] involving attack of $B_{12r}$ (formed by initial homolysis of cyclopropylmethylcobalamin) on cyclopropylmethylcobalamin. The absence of any significant effect of oxygen on the reaction rules out the bimolecular displacement mechanism since $B_{12r}$ is oxidized to $B_{12a}$ by oxygen.[2] It therefore appears that the most likely mechanism for the rearrangement of cyclopropylmethylcobalamin is a unimolecular free radical mechanism involving homolytic fission of cyclopropylmethylcobalamin to give $B_{12r}$ and the cyclopropylmethyl radical, followed by rearrangement to the 3-butenyl radical and recombination with $B_{12r}$ to give 3-butenylcobalamin. $B_{12r}$ and the cyclopropylmethyl and 3-butenyl free radicals should react efficiently with oxygen; $B_{12r}$ is oxidized to $B_{12a}$ by oxygen[2] and planar free radicals (of which cyclopropylmethyl and 3-butenyl are probably examples) react efficiently with oxygen (a π free radical).[134] The small observed effect of oxygen suggests that $B_{12r}$ and the cyclopropylmethyl/3-butenyl free radicals must be present as a 'radical pair' rather than as free radicals and that

rearrangement occurs with the radical close to the cobalt atom.

In order to obtain more information on whether an ionic or free radical pathway is operative the effect of change of solvent was determined. The solvents used were water and methanol, (both of which are protic solvents and strongly solvating) dimethylformamide (which is moderately solvating with a bias towards solvation of cations) and acetic acid (which is mainly dimeric and has lesser polarity and solvating power).[60b] According to a simple, qualitative theory of solvent effects on reaction rates which considers only pure electrostatic effects between ions or dipolar molecules and solvent molecules in the initial and transition states, reactions involving carbonium ions as intermediates should be accelerated by increasing solvent polarity, while those involving free radicals as intermediates should be affected very little or not at all.[60b,132] The change of solvent had only a small and non-systematic effect on the rate of the reaction, suggesting that the intermediates in the reaction are free-radical rather than ionic. However, ionic intermediates cannot be ruled out because the cyclopropylmethyl ligand is well shielded from the external environment by the side-chains of the corrin ligand.

The conversion of cyclopropylmethylcobalamin to 3-butenylcobalamin can be considered as a simple, 1,3 shif of cobalt




and very little movement of the ligand would be required for the conversion. A reaction of this type would not require complete homolysis of the Co-C bond and would probably occur with a lower energy of activation than true homolytic fission. The cobalt atom and the carbons marked with an asterisk would be completely shielded from the solvent by the rest of the ligand and the side-chains of the corrin ligand so that even an incipient $B_{12s}$/ carbonium ion pair would not be affected by change of dielectric constant of the solvent. This type of 1,3 shift would be extremely rapid, allowing no time for reaction of oxygen. Electron spin resonance spectroscopy (ESR) of the isomerase enzyme reactions shows interaction of $B_{12r}$ and the substrate free radical over a distance of 10Å.[135-139] During the cyclopropylmethylcobalamin rearrangement the Co-C bond is lengthened but probably not completely broken and cyclopropylmethyl is strongly influenced by the cobalt atom. Under these conditions it is difficult to say how much of $B_{12s}$/carbonium ion character is mixed into the transition state and it is premature to try to describe the mechanism of rearrangement.

For the alkylcobalamins studied in this thesis there is a correlation between the spectra and equilibria of the cobalamins and their reactivity. Cyclopropylmethylcobalamin does not fit

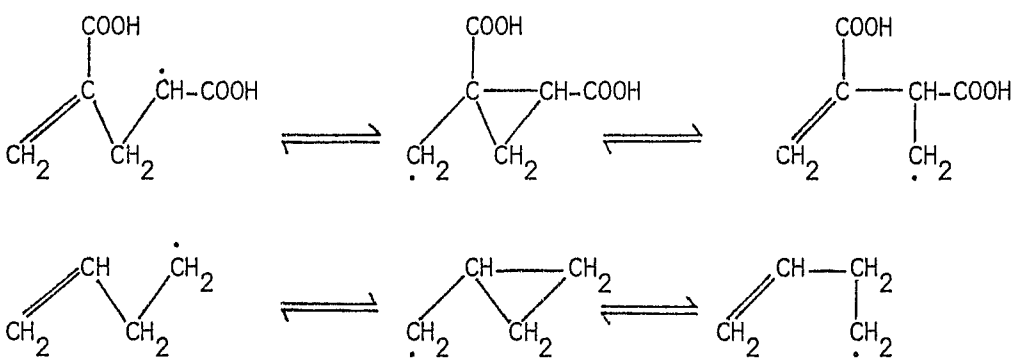
into this pattern; the spectra and pKa's of cyclopropylmethylcobalamin are similar to those of ethylcobalamin (Sections 4.2 and 4.3) but the reactivity of cyclopropylmethylcobalamin is much greater than that of ethylcobalamin. Protonation and removal from coordination of the benzimidazole base of cyclopropylmethylcobalamin has very little effect on the rate of rearrangement of cyclopropylmethylcobalamin, in contrast to reactions for other alkylcobalamins. This suggests that for cyclopropylmethylcobalamin a much smaller lengthening of the Co-C bond is required than for other reactions of alkylcobalamins and there is probably some residual interaction between cobalt and cyclopropylmethyl during the rearrangement.

Publication of the above results on the rearrangement of cyclopropylmethylcobalamin[129] led to the discovery by other workers[133,140] of related reactions with cobaloximes; mechanisms involving homolytic bimolecular displacement[133] and a five-coordinate homoallylic species[140] have been postulated but not proven.

8.4.2 The cyclopropylmethylcobalamin rearrangement as a model for enzymatic carbon-skeleton rearrangements

Of the three enzymatic carbon-skeleton rearrangements it is possible to envisage either a free-radical or carbonium ion mechanism for α-methyleneglutarate mutase but difficult to envisage any mechanism other than a carbonium ion mechanism for glutamate mutase and methylmalonyl-coenzyme A mutase. There are obvious parallels between the rearrangement catalysed by

α-methyleneglutarate mutase and the interconversion of 3-butenyl isomers via a cyclopropylmethyl intermediate.



as pointed out by other workers.[26,133,141-143] The observation that formation of the Co-C bond in the cyclopropylmethylcobalamin model rearrangement inhibits the rearrangement suggests that rearrangement of the substrate while attached to the cobalt during the enzyme reactions is unlikely.

## CHAPTER 9 - STERIC EFFECTS ON HOMOLYSIS OF ADENOSYLCOBALAMIN DURING THE ENZYMATIC ISOMERIZATION REACTIONS

In this chapter the homolytic step of the mechanism of isomerase enzymes is discussed in the context of steric effects in organocobalamins. In previous chapters the steric interactions of the upper axial ligand with the cobalt atom and the corrin ring were studied and a picture built up of the effect of these steric interactions on the spectra, equilibria and reactions of organocobalamins. The study of organocobalamins was confined to adenosylcobalamin (ie. the coenzyme itself) and to simple alkylcobalamins without heteroatoms. These seemed to have the greater applicability to the enzymatic step involving labilization and homolysis of the Co-C bond since the heteroatoms on the sugar moeity do not appear to be necessary for catalytic activity of adenosylcobalamin.[14,144,145]

### 9.1 Neopentylcobalamin as a model for the homolytic fission of adenosylcobalamin during the enzymatic isomerization reactions

As discussed in Chapter 1 the first step in the enzyme reactions catalysed by adenosylcobalamin is the breaking of the Co-C bond to give $B_{12r}$ and the adenosyl free radical. The Co-C bond dissociation energy of primary organocobalamins has been estimated, by comparison with the Co-C bond dissocation energies of organocobaloximes, to be in the range 20-30k cal $mol^{-1}$ [146] which is compatible with the role of adenosylcobalamin as an adenosyl

radical precursor in the enzyme mechanism. Theoretically the equilibrium

$$\text{Co-R} \underset{K_r}{\overset{K_f}{\rightleftharpoons}} \text{Co(II)} + \text{R}\cdot$$

can be set up for any organocobalamin. In fact adenosylcobalamin and simple primary alkylcobalamins in general are stable at room temperature and in the dark[5c] ie. the above equilibrium is displaced so far to the left that the free radical is not available as a reaction intermediate. The enzyme must therefore in some way cause $B_{12r}$ and the adenosyl free radical to be formed when adenosylcobalamin is bound to the apoenzyme.

The enzyme could increase the rate of formation of $B_{12r}$ and the adenosyl free radical either by a kinetic or by a thermodynamic method.[147] A kinetic method would require an increase in both $k_f$ and $k_r$ for the above equilibrium. Since $k_r$ for adenosylcobalamin is very high ($\sim 2 \times 10^9 M^{-1}s^{-1}$)[93] there is little that can be done to labilize adenosylcobalamin by a kinetic method ie. by increasing $k_f$ and $k_r$ but not the equilibrium constant $K_{eq}$. The effect of the enzyme must therefore be to increase $k_f$ without significantly affecting $k_r$, hence increasing $K_{eq}$ for the above equilibrium. The enzyme thus causes the homolytic fission of adenosylcobalamin by a thermodynamic rather than a kinetic method.[147]

It has been suggested that the major shift in $K_{eq}$ is caused by a change in the conformation of the protein at the active site, which is triggered by the binding of the substrate and which is reversed when the substrate has reacted and diffused away.[147]

The necessity for the binding of substrate to the enzyme before the Co-C bond can be labilized to homolytic fission ensures that homolytic cleavage occurs only when the substrate is in position to interact with the active site and prevents unwanted side-reactions.[147] It has been proposed that the change in conformation of the protein results in a distortion in the cobalt coordination sphere[147] ie. distortion of the Co-$\hat{C}_{\alpha}$-$C_{\beta}$ bond angle and lengthening of the Co-C bond.

The mechanism of labilization of adenosylcobalamin homolytic fission by steric distortion of the cobalt coordination sphere is plausible in terms of the experimental evidence available for the enzyme reactions. Adenosylcobalamin in the enzyme-coenzyme-substrate complex (eg. for dioldehydrase) is decomposed by oxygen,[148] showing the some $B_{12r}$ and free radical is formed. It appears that the apoenzyme binds adenosylcobalamin at two sites viz. the side-chains of the corrin ring and the adenyl group of adenosine.[14] Modification of the side-chains of adenosylcobalamin prevents the attachment of adenosylcobalamin to the active site of the enzyme eg. the activity of dioldehydrase is decreased by the conversion of some of the propionamide side-chains of adenosylcobalamin to carboxylic acid, methyl ester or methylamide groups.[149] The adenine ring cannot be replaced by other heterocyclic bases and is required for cofactor activity of adenosylcobalamin.[14] Binding of adenosylcobalamin to the enzyme at two sites makes an ideal situation for distortion of the cobalt coordination sphere

either by 'pulling' on the Co-C bond in order to lengthen it or by distorting the Co-$\hat{C}_\alpha$-$C_\beta$ bond angle.

None of the oxygen atoms in the ribosyl group is essential for catalytic activity since the oxygen of the furanose ring can be replaced by $-CH_2-$ and either of the -CHOH-groups by $-CH_2-$or an acetal group.[14,144,145] It is therefore reasonable to search for models for the homolytic cleavage of adenosylcobalamin among the simple alkylcobalamins.

Neopentylcobalamin, which undergoes homolytic fission at room temperature (Chapter 6), can be used as a model for the homolytic fission of adenosylcobalamin during the enzymatic isomerization reactions. In neopentylcobalamin labilization of the Co-C bond towards homolytic fission occurs because of steric distortion by the organoligand; the Co-$\hat{C}_\alpha$-$C_\beta$ bond angle is increased and the Co-C bond lengthened and weakened. By considering neopentylcobalamin as a model compound for enzyme-bound adenosylcobalamin it can be seen that the postulated mechanism of breaking the Co-C bond of adenosylcobalamin during the enzymatic reactions is feasible and that steric distortion of the cobalt coordination sphere is a reasonable prerequisite for homolysis of the Co-C bond of adenosylcobalamin in the enzyme-coenzyme-substrate complex.

Cyclohexylcobalamin, which decomposes partially by homolytic fission at room temperature (Chapter 7), has its Co-$\hat{C}_\alpha$-$C_\beta$ bond angle decreased and its Co-C bond lengthened due to steric distortion by

the organoligand. This shows that either increasing or decreasing the Co-$\hat{C}_{\alpha}$-C$\beta$ bond angle results in labilization of the Co-C bond to homolytic fission and suggests that the mode of distortion of the cobalt coordination sphere of enzyme-bound adenosylcobalamin may be different for different enzymes and for the same enzyme with different substrates.

Homolytic fission of enzyme-bound adenosylcobalamin occurs much more rapidly (eg. one mole of methylmalonyl-coenzyme A mutase catalyses the conversion of 1 400 - 2 000 moles of succinyl-coenzyme A per minute)[20] than homolytic fission of neopentylcobalamin (Table 6.1). The amount of steric distortion in the coordination sphere of enzyme-bound adenosylcobalamin must be much greater than for neopentylcobalamin but the principle of labilization of the Co-C bond (viz steric distortion) is probably the same in both cases.

In neopentylcobalamin and cyclohexylcobalamin homolysis of the Co-C bond occurs in the six-coordinate, 'base-on' form. In enzyme-bound adenosylcobalamin homolysis probably occurs in the five-coordinate, 'base-off' form since five-coordinate coenzymes, such as 5'-deoxadenosyl-adenylcobamide are active in the enzyme reactions.[150] Steric compression, in alkylcobalamins with bulky axial ligands, is relieved by removal of the benzimidazole base from coordination and movement of the cobalt atom out of the plane of the corrin ring; this probably also occurs in enzyme-bound adenosylcobalamin.

## 9.2 Sterically-distorted adenosylcobalamin as an intermediate in the enzymatic isomerization reactions

The mechanism for the enzyme-catalysed labilization of the Co-C bond towards homolytic fission proposed in section 9.2 involves an intermediate enzyme-bound adenosylcobalamin species in which the Co-$\hat{C}_{\alpha}$-C$\beta$ bond angle is distorted, the Co-C bond is lengthened and the benzimidazole base is not coordinated. On the basis of the study of the effect of steric distortion of the cobalt coordination sphere on the UV-visible spectra of alkylcobalamins (Chapter 4) it is possible to predict that a very strained form of adenosylcobalamin would have a spectrum different from that of normal 'base-off' adenosylcobalamin ($\lambda$= 460nm for the $\alpha\beta$ band) and possibly similar to that of 'base-off' isopropylcobalamin, neopentylcobalamin, cyclopentylcobalamin or cyclohexylcobalamin ($\lambda$= 440nm for the $\alpha\beta$band). $B_{12r}$ which is observed under steady state conditions during the enzymatic reactions[18,148,149,151-154] has peaks at 312nm, 405nm and 473nm in the 'base-on' form and at 315nm, ~405nm and 470nm in the 'base-off' form.[5b] It should therefore be possible to observe the presence of a strained adenosylcobalamin intermediate, in the presence of $B_{12r}$, during the steady state phase of the enzymatic reactions.

Examination of a number of published UV-visible spectra of adenosylcobalamin-requiring enzymes (in the presence of coenzyme and substrate)[18,148,149,151-154] showed that in three cases absorbance bands in addition to those of $B_{12r}$ were present. The three enzymes and their substrates are listed in Table 9.1

together with references to the original papers.

Table 9.1 - UV-visible spectra of isomerase enzymes

| Enzyme | Substrate | Reference |
|---|---|---|
| Glycerol dehydrase | propane-1,2-diol | 18 |
| Dioldehydrase | propane-1,2-diol | 149 |
| Ethanolamine ammonia lyase | l.-2-aminopropanol | 153 |

All these spectra show the following features:-

1) Near complete conversion of adenosylcobalamin to $B_{12r}$ or other forms with low absorbance at 520nm.

2) Absorbance bands at 440-450nm and at approximately 375nm (except for glycerol dehydrase where the spectrum has a sharply rising background below 400nm) which are not present in normal $B_{12r}$.

The UV-visible spectra of the steady state of adenosylcobalamin-dependent enzymes therefore provide good evidence for the presence of some additional cobalamin which absorbs at approximately 440nm and 375nm ie. is not the normal 'base-off' adenosylcobalamin or $B_{12r}$ in the 'base-on' or 'base-off' forms and which could be 'base-off' sterically-strained adenosylcobalamin. A simulated UV-visible spectrum consisting of eighty per cent $B_{12r}$ and twenty per cent cyclohexylcobalamin is very similar to the spectrum of glycerol dehydrase quoted by Foster et al.[18] (Fig.9.1)

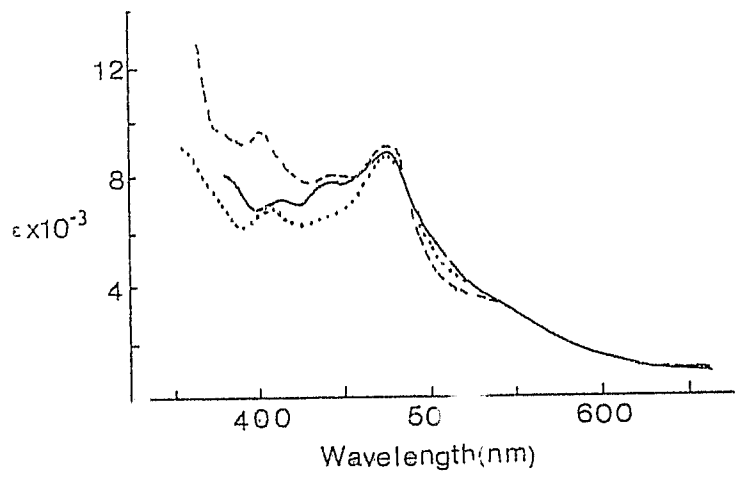



Fig.9.1. Spectra of the enzyme-coenzyme-substrate steady state of glycerol dehydrase (---) and $B_{12r}$ in the base-off form (...) (from ref. 18) and a simulated spectrum consisting of eighty per cent $B_{12r}$ and twenty per cent cyclohexylcobalamin (——).

The evidence from ESR spectroscopy is consistent with the presence of a small amount of a cobalamin other than $B_{12r}$. ESR spectroscopy of the frozen enzyme-substrate complexes showed the presence of a $B_{12r}$ species in the following amounts:- glyceroldehydrase/propane-1,2-diol fifty per cent,[19] dioldehydrase/ propane-1,2-diol forty per cent,[17] ethanolamine ammonia lyase/ aminopropanol sixty-five per cent[14] ie. the amount of $B_{12r}$ measured by ESR is much smaller than that measured by UV-visible spectroscopy. The discrepancy between the results from UV-visible and ESR spectroscopy has been ascribed to interactions between the paramagnetic $B_{12r}$ and an organic free radical[19] but it is possible that some of the discrepancy arises from an overestimate of the

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